Role of TNF in the Regulation of the Innate Immune Response in Leishmaniasis

by

Shanshan Hu

The Menzies Institute for Medical Research

at the University of Tasmania

Master of Medicine

Clinical Pharmacology

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

University of Tasmania

October, 2017

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Signed: Shanshan Hu

Date: 22 September 2017

Authority of Access

This thesis is not to be made available for loan or copying for two years following the date this statement was signed. Following that time, the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Signed: Shanshan Hu
Date: 22 September 2017

Publications

Parts of this thesis have contributed to publications. Listed below are these publications, along with author contributions. In all cases the material included in the thesis were performed by the candidate, except where due acknowledgement is made.

Primary supervisor	Signature	Date
Professor Heinrich Korner		22/09/2017

Chapter 1. Introduction

The literature review is partly based on the published review:

Hu S, Wei W, & Korner H. 2017. The role of monocytes in models of infection by protozoan parasites. *Mol Immunol*. 88:174-184.

Author contributions:

SH and HK wrote the manuscript

WW refined the manuscript

Chapter 3. Absence of TNF leads to liver infection during *L. major*-induced cutaneous leishmaniasis

Chapter 4. The balance between TNF and IL-6 in regulation of monocyte/macrophage differentiation

Chapter 3 and 4 is based on the published paper:

Shanshan Hu, Cameron Marshall, Jocelyn Darby, Wei Wei, A. Bruce Lyons, and Heinrich Korner. Absence of TNF supports alternative activation of macrophages in the liver after infection with *L. major*. *Front Immunol.* 2018 Jan 19;9:1.

Author contributions:

SSH & HK designed the experiments

SSH & CM performed sample collection and laboratory work

SSH performed laboratory analysis and prepared the figures

SSH & HK drafted the manuscript

Acknowledgements:

Terry Pinfold and Jocelyn Darby for flow cytometry analysis

David Steel and Sarah Kane for AQIS work

Peta Lawrie for animal experiments

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University

University of Tasmania Animal Ethics Committee permit numbers: A0013934, A0013935.

Signed: Shanshan Hu

Date: 22 September 2017

Conference presentations

Part of the work contained in this thesis has been presented at international conferences as detailed below:

Shanshan Hu, Cameron Marshall, Jocelyn Darby, Bruce Lyons, Heinrich Korner. Potential Role of IL-6 in TNF-Medicated Monocyte/Macrophage Differentiation during Cutaneous Leishmaniasis. 2017. 16th TNF Superfamily Meeting. Singapore. Oral Presentation.

Shanshan Hu, Cameron Marshall, Jocelyn Darby, Bruce Lyons, Heinrich Korner. 2016. TNF deficiency leads to splenomegaly following cutaneous infection with Leishmania major. 16th International Congress of Immunology 2016. Australia. Poster presentation.

Acknowledgements

The completion of my PhD has been a long and challenging journey fulfilled with happiness and tears, and I am elated and proud that I have finished. However, I could not have succeeded without the help of many people. I hope my acknowledgment does justice to the support that I have received and the full extent of my gratitude.

First of all, I would like to thank my supervisors, Prof. Heinrich Korner and Dr. Bruce Lyons, for their supports and guidance of my PhD. I feel very fortunate having had the opportunity to spend my last four years on this project. The knowledge and skills I learned from them are the life-long fortune and will affect me in the future study and work.

I would also like to thank Ms. Jocelyn Darby and Dr. Terry Pinfold for their support. They gave me the best professional support for the experiments and helped me to improve my spoken and written English all the time. Also, their encouragement, consolation, and affirmation helped me get through difficult times. I could not have finished my PhD without you.

Furthermore, I am really grateful to Menzies Institute for Medical Research, at University of Tasmania, for their professional support, which inspired and encouraged me to present my research in international conferences. Meanwhile, I would like to thank my coordinators, A/ Prof. Tracey Dickson, Dr. Costan Magnussen and Dr. Joy Rathjen, for their support and help. Also, I would like to thank Dr. David Steel, Sarah Kane, Peta Lawrie as well as the people from Cambridge Farm Facility, for my AQIS and animal work support. I would like to thank

Prof. Ding and Anhui Medical University, for offering such a good study opportunity and living experience in Tasmania.

Finally, I would like to sincerely thank my family and my friends for their encouragement and understanding. Thanks for my parents' great and endless love. To my husband, Dr. Di Wang, I could not have finished my PhD without your love, understanding, and support. Thank you.

Table of contents

List of Figures

List of Tables

List of Abbreviations

Arg-1: Arginase-1

B6.TNF$^{-/-}$: B6.WT mice lacking of TNF gene

B6.WT : C57BL/6 background

CBA: Cytometric bead arrays

CCR2: C-C chemokine receptor type 2

cDCs: Conventional DCs

CDP: Common DC precursor

cMoPs: Common monocyte progenitors

CR: Complement receptor

CSF1 or M-CSF: Colony-stimulating factor 1

CSFR, M-CSFR or CD115: Macrophage colony-stimulating factor receptor

CX3CR1: CX3C chemokine receptor 1

DCs: Dendritic cells

EDTA: Ethylenediaminetetraacetic acid

GM-CSF: Granulocyte macrophage colony Stimulating factor

H&E: Haematoxylin and eosin

HSCs: Hematopoietic stem cells

IL: Interleukin

IL-4R: IL-4 receptor

iNOS: Inducible nitric oxide synthase

IFN-γ: Interferon γ

JAK: Janus activated kinase

L: Leishmania

MDP: Monocyte/macrophage and dendritic cell Progenitors

mIL-6R: Membrane-bound receptor IL-6R

mins: Minutes

Mo-M: Monocyte-derived macrophages

Mo-DCs: Monocyte-derived DCs

MPS: Mononuclear phagocyte system

NK: Natural killer

NO: Nitric oxide

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

pDCs: Plasmacytoid DCs

PALS: Periarteriolar lymphoid sheaths

ROS: Reactive oxygen species

RA: Rheumatoid arthritis

SEM: Standard error of the mean

sIL-6R: Soluble form of IL-6R

SLE: Systemic lupus erythematosus

STAT: Signal transducer and activator of transcription

TBS: Tris buffered saline

TGF-β: Transforming growth factor-β

Th1: T helper 1

Th2: T helper 2

TNF: Tumor necrosis factor

TNFR1 or TNFp55: TNF receptor-1

TNFR2 or TNFp75: TNF receptor-2

Abstract

The absence of tumor necrosis factor (TNF) causes lethal infection by *Leishmania major* (*L. major*) in normally resistant mice C57BL/6J (B6.WT) mice, but the underlying mechanism has so far remained elusive. We found B6.WT mice without the TNF gene (B6.TNF$^{-/-}$) displayed not only non-healing cutaneous lesions but also serious liver and spleen infection upon *L. major* infection. The enlarged liver showed increased inflammation and harbored a new monocyte-derived-macrophage population showing a CD45^{+}F4/80^{+}Ly6C^{low}CD11b^{hi} phenotype. This population continuously accumulated and typically displayed a M2 macrophage phenotype with high expression of CD206, Arginase-1 and interleukin-6 (IL-6). Of note, IL-6 is normally considered as a pro-inflammatory cytokine, but it also has been shown to have anti-inflammatory properties. Absence of TNF induced increased IL-6 expression and upregulated M-CSF receptor expression downstream. The elevated IL-6 level skewed monocyte differentiation from dendritic cells (DCs) to macrophages. Furthermore, TNF inhibited both IL-6-induced gp130-STAT3 and IL-4-STAT6 signaling activation, thereby abrogating an IL-6 facilitated M2 macrophage polarization.

Spleens from B6.TNF$^{-/-}$ mice were around three times larger than B6.WT mice and contained significantly more parasites. Plasmacytoid dendritic cells (pDC) are the key immune inducing population during leishmaniasis, producing IL-12 and promoting a Th1 immune response. B6.TNF$^{-/-}$ mice displayed significantly fewer pDC and a non-defined B220^{+} monocyte-derived cell population compared to B6.WT mice. T cells and B cells did not show significant differences between the two strains, but CD11b^{+}F4/80^{+} cells were found to be in higher numbers in B6.TNF$^{-/-}$ mice.

Therefore, our results set the basis to investigate the role of IL-6 signaling in macrophage polarization. Our findings also demonstrated the critical role of TNF in the development of different DC populations, that may subsequently affect T cell-mediated protection against *L. major* infection. In conclusion, these findings may potentially explain the increased risk of opportunistic infection and relapses in patients receiving anti-TNF treatment.

Chapter 1.
Introduction

1.1 Leishmaniasis

Leishmaniasis is a parasitic disease caused by protozoan genus *Leishmania* that is transmitted to mammalian hosts by an infected sand fly. This disease results in an estimated 1.3 million new cases and more than 30 000 deaths annually [1]. Due to an inadequate knowledge of the parasite as well as the complexity of the immune response, there is no vaccine to prevent *Leishmania* infection and controlling the disease is still based on chemotherapy. Therefore, understanding leishmaniasis and its interaction with the host, are of key importance for developing better interventions for this disease. The control of *Leishmania* infection in the mammal host is mainly mediated by the cellular immune response, and especially the innate immune response such as monocyte/macrophage activation. In this chapter, all the information of leishmaniasis, the immune response and specifically the importance of monocytes for the control of leishmaniasis will be reviewed and discussed.

1.1.1 *Leishmania* life cycle

Leishmaniasis is a vector-borne disease, which can be easily transmitted by the bite of infected female sand flies. Infected host cells are ingested by the sand fly during its blood meal and then the amastigotes are released which replicate extracellularly in the digestive tract of the sand fly as actively motile, flagellated promastigotes. When mammals are bitten by an infected insect vector, *Leishmania* can be inoculated into the mammals' skin through the proboscis during the blood meal. Promastigotes interact with leukocytes such as neutrophils, which are the first population recruited to the site of the bite, and infected neutrophils act as "Trojan Horses" that help parasite to silently enter into macrophages [2].

Although neutrophils were found to harbor most of the parasites, parasites do not differentiate from promastigotes into amastigotes until they transfer into the macrophages. They lose their

flagella, reduce their size and become adapted to the low pH and hydrolase-rich environment within the phagolysosomes. Amastigotes propagate in macrophages and rapidly infect surrounding macrophages, making the mammal a reservoir host.

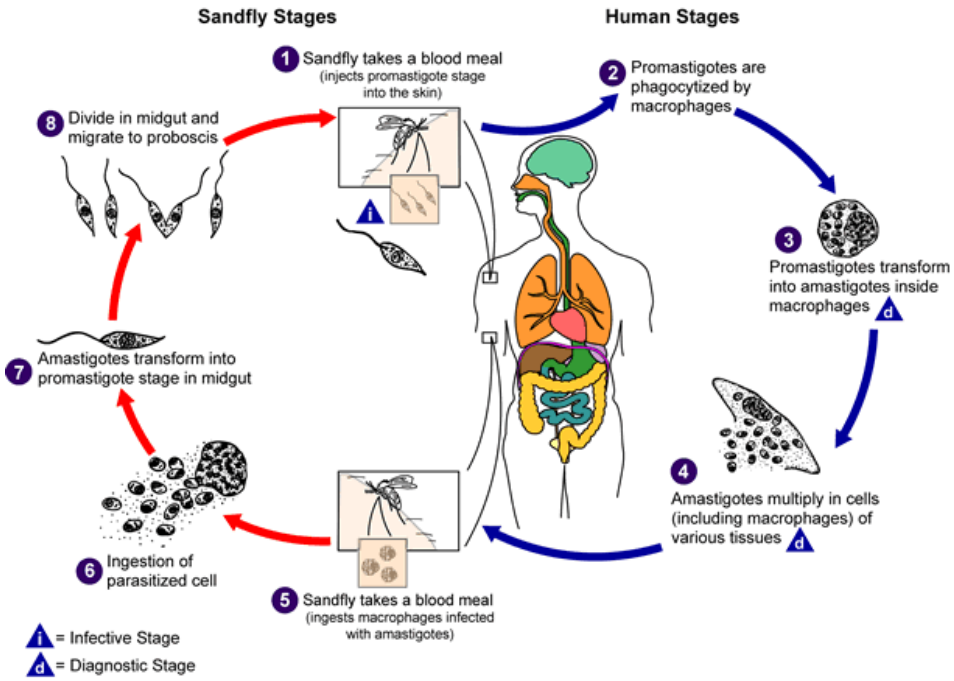


Figure 1.1 The life cycle of *Leishmania* parasites

Sandfly bites host and seed promastigotes into the skin through the proboscis (1). Recruited macrophages engulf the promastigotes (2), which then turn into amastigotes inside the macrophages (3). Amastigotes reproduce, burst out and infect surrounding cells (4). Amastigotes are taken by a sand fly in a blood meal (5-6), and then transform into promastigotes in the gut of vector (7) and eventually differentiate into metacyclic promastigotes which migrate to the proboscis and can viably infect another vertebrate host.

Reproduced from Centers for Disease Control and Prevention (2013) and content source from Division of Parasitic Diseases of Globe health

1.1.2 Symptoms of leishmaniasis

Humans infected with *Leishmania* generate a pleomorphic syndrome in which symptoms vary depending on the species of *Leishmania*. More than 20 species of *Leishmania* have been identified as being involved in humans causing symptoms ranging from self-healing skin lesions to fatal visceral organ infection. Cutaneous leishmaniasis, often caused by *Leishmania (L). major, L. tropica, L. amazonensis* and *L. mexicana*, is the most common form of leishmaniasis and accounts for more than 50% of new cases. It normally causes skin lesion and ulcers, and patients will become a potential carrier and transmitter with life-long scars. Visceral leishmaniasis is a type tightly associated with a fatal form of the infection. It is known as "kala azar", and caused by *L. donovani, L. infantum, L. tropica and L. amazonensis,* but humans are the only known hosts for *L. donovani*. Patients with visceral leishmaniasis can be asymptomatic or have intermittent fever, splenomegaly and hepatomegaly, and it is lethal if left untreated. Mucocutaneous leishmaniasis is caused by *L. braziliensis, L. panamensis and L. guyanensis* and can induce an extensive infiltration damage of mucous tissues in the nose, mouth and throat, and eventually destruction of cartilage.

Figure 1.2 Different forms of leishmaniasis. Reproduced from WHO (2013)

Cutaneous leishmaniasis (A), Visceral leishmaniasis (B) and mucocutaneous leishmaniasis (C).

Pictures were acquired and downloaded from A http://www.who.int/leishmaniasis/cutaneous_leishmaniasis/en/, B http://www.who.int/campaigns/world-health-day/2014/photos/leishmaniasis/en/, and C http://www.who.int/leishmaniasis/mucocutaneous_leishmaniasis/en/.

1.1.3 Immune response during leishmaniasis

The control of *Leishmania* infection is mainly mediated by cellular immune responses including both innate and adaptive immunity. Antigen-presenting cells produce interleukin (IL) -12 that primes interferon γ (IFN-γ) secreting T helper 1 (Th1) cytokines, and in turn, induces macrophage activation. Activated macrophages produce nitric oxide (NO) and reactive oxygen species (ROS), both highly effective mechanisms for killing *Leishmania*. Although a strong humoral response is observed during leishmaniasis, antibodies do not contribute to protection. Moreover, production of pro-inflammatory and anti-inflammatory cytokines not only directly participates in parasite killing but also controls the immune response, determining the outcome of the disease.

1.1.4 Immune response in cutaneous leishmaniasis

To better understand the immune response against leishmaniasis, studies performed on mice have been the major models. Experimental cutaneous leishmaniasis has been widely used for the last 40 years and is based on a subcutaneous inoculation of mice with isolates of the species *L. major*. Outcomes of the infection feature a strong genetic dichotomy, with mice of a C57BL/6 background (B6.WT) displaying a localized, self-healing infection characterized by IFN-γ production, while mice of a BALB/c background respond with a preferential production of T helper 2 (Th2) cytokines, such as IL-4, IL-10 and IL-13, and after a progressive course of disease and a marked visceralization of the pathogen, invariably succumb to the infection. This genetic dichotomy as the basis of resistance or susceptibility was instrumental in the development of the Th1/Th2 paradigm [3], and its correlation led to the concept that the balance of Th1 and Th2 determines the outcome of infection.

Macrophages play very important roles in *Leishmania* infection because they are the host cells for parasite survival. Macrophage phagocytosis of *Leishmania* parasites depends on receptor recognition, including complement receptor (CR)3, mannose receptor and fibronectin receptor. Although individual ligation of these receptors allows macrophages to take up parasites, it fails to trigger the activation of macrophages [4]. For example, single CR3 ligation is not able to trigger nicotinamide adenine dinucleotide phosphate-oxidase oxidase activation in macrophages [5]. Thus, *Leishmania* preferentially use CR3 to "silently" enter into macrophages and enhance their intracellular survival. Therefore, infected macrophages can act as reservoir for resident parasites and replication occurs until they die, which sets free the parasites and leads to the infection of surrounding cells [6].

Although *Leishmania* takes advantage of phagocytosis to enter into macrophage phagolysosomes and subsequently propagate, macrophages are also the main effector cells that kill the parasites. Macrophages can be activated by different signals and are endowed with different functions that subsequently can determine distinctive outcomes of disease. Macrophage activation generally can be categorized into two different types, classical and alternative activation. Classically activated macrophages (also referred to as M1 macrophage) are induced by the presence of Th1 cytokines such as IFN-γ, and are able to produce pro-inflammatory cytokines and cytotoxic mediators, typically inducible nitric oxide synthase (iNOS). The enzyme iNOS promotes the oxidation of the terminal guanidine nitrogen of L-arginine in macrophages and then generates NO, which is a toxic mediator known for killing *Leishmania* parasites. iNOS-dependent NO production is the major mechanism responsible for protection against *Leishmania* infection. This has been demonstrated by inhibition of NO *in vitro* [7] and by the investigation of an increased susceptibility of iNOS-deficient mice to the infection with *L. major* [8]. Moreover, it has been demonstrated that the pro-inflammatory

cytokine TNF provides a second signal to to enhance NO production of IFN-γ -activated macrophages against *L. major* [9]. In contrast, Th2 cytokines IL-4, IL-10 and IL-13 cause an alternative activation of macrophages (referred to as M2 macrophages) [10]. Alternatively activated macrophages are normally recognized as anti-inflammatory and involved in mitigating and resolving the inflammatory response. They highly express CD206, but also produce a large amount of arginase-1 (Arg-1), which competitively hydrolyzes L-arginine to ornithine and urea. This inhibits arginine utilization for NO synthesis and reduces the pro-inflammatory response. Moreover, ornithine contributes to polyamine and proline synthesis pathways, which are very important for cellular proliferation and tissue repair. Of note, this process inhibits NO production, directly impairing NO-mediated parasite elimination and facilitating the parasite replication in M2 macrophages [11]. Therefore, appropriate activation of macrophages is crucial for determining the outcome of cutaneous leishmaniasis.

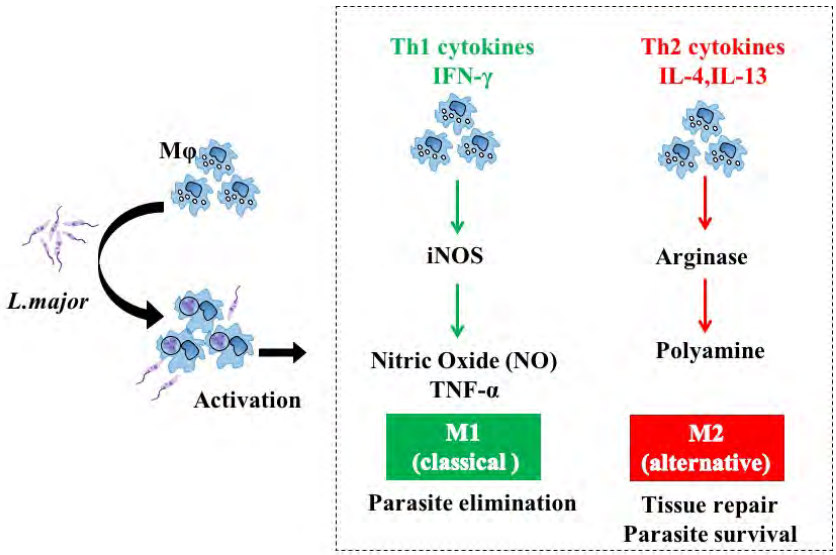


Figure 1.3 Classical and alternative activated macrophage

During *L. major* infection, macrophages can be activated by Th1 cytokines such as IFN-γ (classical activated macrophage or M1 macrophage), and then induce iNOS production and generate NO for their microbicidal actions. In contrast, alternative activation or M2 activation can be triggered by IL-4 and IL-13. It catalyzes L-arginine to

Arg-1 then synthesize the proline to re-build the damaged tissue, therefore helps tissue repair. However, high Arg-1 production will also facilitate parasite survival.

In addition to macrophages, cells such as neutrophils and monocytes also participate in shaping the immune response to infection. The role of neutrophils during cutaneous leishmaniasis is complex and multi-faceted. As mentioned above, neutrophils can act as "Trojan Horses" to help parasite invasion and survival. Reduced neutrophil recruitment has been shown to be associated with decreased lesions during leishmaniasis [12]. Phagocytosis of infected neutrophils by macrophages and dendritic cells (DCs) inhibits activation of these cells [13]. Furthermore, macrophages secrete the anti-inflammatory cytokine transforming growth factor-β (TGF-β), a cytokine which promotes alternative macrophage activation, thereby facilitating that parasites enter silently into macrophages and replicate [14]. Although neutrophils have also been reported to kill parasites by proteolytic enzymes, reactive oxygen species [15, 16], and neutrophil extracellular traps *in vitro* [17], these mechanisms have been shown to be insufficient in controlling infection depending on the species of *Leishmania*. In a later phase of the infection monocytes are recruited to the site of infection and become the dominant cells that interact with *Leishmania*. Key aspects of monocyte biology, relevant to this thesis, are described in the following sections.

1.1.5 Innate immune response in visceral leishmaniasis

In visceral leishmaniasis parasites disseminate from the site of infection in the skin to propagate in cells of liver, spleen and bone marrow. Most people infected with visceral *Leishmania* species experience an asymptomatic infection, and only a small number of people will develop a clinically severe infection. Human immunodeficiency virus infected patients or patients suffering from an autoimmune disease such as rheumatoid arthritis (RA) or systemic lupus

erythematosus (SLE) receiving immune suppressive anti-TNF therapy, showed an increased risk of infection, indicating the importance of the role of the host immune response against leishmaniasis. Therefore, in order to better understand the immune response against leishmaniasis, experimental murine models were analysed. Although susceptible strains such as BALB/c mice exhibit a long-term chronic infection rather than a fatal outcome as found in humans [18], the mouse model provides an opportunity to investigate organ-restricted immunity, and potentially offers treatment insights.

Liver is one of the targets of infection in visceral leishmaniasis. The infection is self-resolving and depends on T cell-mediated immunity and the formation of granulomas [19]. In the early stages of infection, parasites reach the liver through the portal vein and reside as amastigotes in liver resident macrophages, the Kupffer cells [20]. Kupffer cells harbor most parasites in the liver and exhibit a reduced capacity to kill the parasites once they are infected. Therefore, hepatic parasite control depends primarily on granuloma formation. Liver granulomas were found increased in number and size in order to facilitate clearance of parasites and resolution of infection [21]. Granulomas consist of a core of fused and infected Kupffer cells with outer layers of mononuclear cells containing recruited monocytes, $CD4^+$ and $CD8^+$ T cells and other immune cells [22]. This provides an inflammatory focus, limiting hepatic infection and facilitating effective collaboration among different cells which is required for parasites elimination. Monocytes and neutrophils are recruited to the liver following cytokine and chemokine secretion by infected Kupffer cells. Depletion of monocytes or neutrophils during visceral leishmaniasis delayed granuloma formation and aggravated disease indicating their importance in parasite control [23]. Recruitment of monocytes and neutrophils is followed by recruitment of T cells, which is essential for an efficient granuloma response. In particular, $CD4^+$ T cells are important because they produce high levels of pro-inflammatory cytokines

including tumor necrosis factor (TNF), IFN-γ, and lymphotoxin-α, that can further promote cell recruitment and support active antimicrobial responses [24].

Of note, the effectiveness of hepatic granulomas depends on their degree of maturation. Only mature granulomas were found to efficiently kill parasites [20]. A threshold level of TNF is required for granuloma formation and maintenance. This was demonstrated in a mouse model of *M. tuberculosis* infection. If granulomas failed to assemble in the lungs of mice deficient for TNF, *M. tuberculosis* infection remained uncontrolled [25, 26]. Although cells continually infiltrated, they failed to collaborate as granulomas without TNF signaling [27], causing a fatal outcome. Furthermore, a series of chemokines and cytokines which mediate cell migration were also reduced due to lack of TNF, leading to a reduction in the ability of the cells to aggregate and form granulomas [28, 29]. During visceral leishmaniasis, lack of TNF resulted in absence of early granuloma development and an increased liver parasite burden [30]. However, administration of TNF was found to be of no extra benefit in enhancing macrophage activity and anti-leishmanial function. In contrast, excess TNF paused the process of granulomas formation and exacerbated infection [31]. Interestingly, anti-inflammatory cytokines such as IL-4 or IL-13 have also been found to participate in the formation of granulomas during visceral leishmaniasis. BALB/c mice deficient for the IL-4 receptor (IL-4R) or IL-13 showed not only increased parasitic burden but also less granuloma maturation [32].

Experimental infection with *L. donovani* is characterized by a different organ-specific immune response. Parasites multiply rapidly in the early stages of infection in the liver, but they are controlled by the cell-mediated immune response and granuloma formation [33]. However, the infected spleen fails to clear the parasites and a life-long, chronic infection persists during visceral leishmaniasis. Initially, the splenic structure is intact in order to generate the early

immune response. Marginal zone macrophages and marginal metallophilic macrophages are the primary means to clear parasites from blood flow. It has been reported that marginal zone macrophages kill 50% of the initial parasite inoculum within the first 24 hours after infection [34]. DCs acquire *Leishmania* antigen in the marginal zone, subsequently migrate to the T cells area, mature and begin to produce IL-12 to initiate a T cell response in the periarteriolar lymphoid sheaths (PALS) [35, 36]. $CD4^+$ T cells producing IL-12 induce the production of the Th1 cytokine IFN-γ and stabilize the Th1 lineage which contributes to the effective response against visceral leishmaniasis [37]. The encounter between DCs and T cells is very important not only for the spleen itself but also contributing to the immune defence in the liver. Impaired DC migration and less IL-12 production were tightly associated with reduced effector T cell migration from spleen to liver, leading to impaired granuloma formation [36]. $CD8^+$ T cell was found increased after infection. They upregulated T-bet and eomesodermin in response to IL-12, promoting expression of TNF and IFN-γ, in turn, allowed $CD8^+$ T cell to act as effector cells to kill the parasites [38]. Splenic macrophages can be activated by $CD8^+$ T cell-derived IFN-γ, which has been proved effective in killing the parasites as well [39].

In the initial phase, parasite replication is controlled in the spleen. The splenic structure is intact and the T cell-mediated immune response is still detected. However, this response cannot eliminate the parasites from the spleen effectively, and the parasites persist and begin to increase after three weeks of infection. The disease progression goes into the chronic stage, characterising with striking splenomegaly. Considering the increased size and weight of the spleen, a number of changes of splenic architecture occurs, leading to an immunocompromised status. It is reported that disorganization of white pulp and the marginal zone are observed during splenic leishmaniasis. Especially in the while pulp, the collapse of germinal centre and PALS impaired the T cell function [40]. TNF is the key mediator which induces

disorganization of the structure. Increased TNF is responsible for disruption of the splenic marginal zone in visceral leishmaniasis [41], which is similar to the developmental abnormalities in mice lacking TNF cytokines [42]. The marginal zone is the compartment where DCs interact with T cells and induce an antigen-specific T cell response. As a consequence, T cell priming was diminished [41]. Similarly, TNF decreased $gp38^+$ cells and mediated PALS disorganization. These cells produce chemokines such as CCL19 and CCL21 to attract T cells, that are important for establishment and maintaining PALS [43-45]. Additionally, immunological dysfunction was associated with the chronic inflammatory state during visceral leishmaniasis. High levels of IL-10 were found in the spleen in visceral leishmaniasis, which has been suggested to reduce the tissue damage caused by the chronic inflammation. However, it also deactivated the leishmanicidal effect. IL-10 receptor blockade increased the secretion of IFN-γ, promoted parasite killing and enhanced murine resistance to visceral infection [46, 47].

1.2 Monocytes

Monocytes are highly plastic and heterogeneous. They were once thought to be the precursor cells of all tissue resident macrophages. However, it is now accepted that most tissue resident macrophages are derived from embryonic precursors and maintain themselves by self-renewal, independent of monocyte recruitment. Monocytes play very important roles in the inflammatory response, including parasitic infection. They are rapidly recruited to the site of pathogen insult, differentiating into inflammatory cells, but can also differentiate into anti-inflammatory subsets, depending on the cytokine environment. Therefore, there is a need to update and summarize our knowledge of monocytes and their function in infectious diseases. Therefore, it is very important to discuss the evidence that has dramatically changed our

understanding of monocyte and macrophage development and the potential of manipulating these cells as therapeutic agents in infectious diseases.

1.2.1 Origins of monocytes

The mononuclear phagocyte system (MPS) was defined as a specialized system of phagocytic cells by Ralph van Furth et al. in 1968 [48, 49]. It included only monocytes and macrophages until the 1980s, when DCs were also incorporated into MPS after Steinman identified them as being a distinct cell subset [49, 50]. Monocytes are a critical part, acting as precursors of tissue macrophages able to replenish them during adulthood. However, this old dogma has been modulated since fate mapping results revealed that tissue resident macrophage, such as Kupffer cells, lung alveolar and splenic macrophages, are established prior to birth and their replenishment is independent of monocyte input [51].

Monocytes have been shown to exist in the fetal circulation when hematopoietic stem cells (HSCs) have seeded the fetal liver [51]. Hematopoietic stem cells then give rise to common myeloid progenitors, granulocyte-macrophage precursors and monocyte/macrophage and dendritic cell progenitors (MDP), which in turn commit to common monocyte progenitors (cMoPs) restricted to monocytes and macrophages or dedicated toward a common DC precursor (CDP). CDP are not able to give rise to monocytes but are the precursors for classical DCs in the bone marrow. During inflammation, monocytes can be recruited through blood to the site of infection, giving rise to subsets of inflammatory macrophages and DCs that share many phenotypic features and functions.

In mice, monocytes represent around 4% of nucleated cells in the blood [52] and in spleen, blood and bone marrow are generally identified as $CD11b^{+}CD115^{+}$ [53, 54]. Their

development and survival is dependent on colony-stimulating factor 1(CSF1 or M-CSF), and mice depleted of CSF or its receptor CSFR (also known as M-CSFR or CD115) showed reduced numbers of blood monocytes [55, 56]. In the blood, it is now accepted that this $CD11b^{+}CD115^{+}$ cell population is subdivided into two main monocytic populations, $Ly6C^{hi}$ in mice and $Ly6C^{lo}$, based on Ly6C expression on the cell surface. $Ly6C^{hi}$ cells are defined as classical monocytes with $CCR2^{hi}CX3CR1^{lo}$ expression.

The classical $Ly6C^{hi}$ monocytes represent approximately 2-5% of circulating white blood cells in mouse which can be recruited rapidly to sites of infection or inflammation [57], where they extravagate and give rise to monocyte-derived macrophages (Mo-M) or monocyte-derived DCs (Mo-DCs). These cells are equipped with toll like receptors and scavenger receptors, acting as phagocytes recognizing pathogen-associated molecular patterns and clearing the microorganisms. Following infiltration, they also produce inflammatory cytokines, myeloperoxidase, and superoxide and thus contribute to the inflammation [53, 58, 59].

$Ly6C^{lo}$ monocytes have a longer half-life than two days which can be prolonged to 2 weeks in the absence of a renewal from $Ly6C^{hi}$ monocytes. Thus, $Ly6C^{lo}$ monocytes were considered as terminally differentiated blood-resident, rather than monocytes, which complemented macrophages and DCs [60]. However, they are now considered to patrol and survey endothelial integrity. Using intravital microscopy, it was revealed that $Ly6C^{lo}$ monocytes exhibited a constitutive long-range crawling on the luminal side of the endothelium in the steady state. They patrol the blood vessel, engulfing dying cells and debris, and participate in the early defence against to the insult as well as suppressing T cells responses to maintain homeostasis [60, 61].

During inflammation, patrolling Ly6C^{lo} monocytes were shown to develop a very early but transient inflammatory response. They extravasated rapidly from blood vessels to the peritoneal cavity within 1 hour after infection, and produced an initial burst of TNF [62]. Ly6C^{hi} monocytes preferentially initiated DC-like cells in response to *Listeria monocytogenes* infection. Compared to Ly6C^{hi} cells, Ly6C^{lo} monocytes are characterized as macrophage-like cells, more likely to produce anti-inflammatory cytokines such as IL-10 and IL-4, highly express Arg-1 and differentiate into M2 macrophages that contribute to the tissue repair [60]. It was found that Ly6C^{lo} monocytes highly expressed vascular endothelial growth factor to promote myocardium healing after mobilized Ly6C^{hi} monocytes mediated inflammation [63].

Intriguingly, the conversion between Ly6C^{hi} and Ly6C^{lo} monocytes has been recently reported. In the steady state, Ly6C^{hi} monocytes were found to return to the bone marrow and differentiate into Ly6C^{lo} monocytes to supplement the pool of Ly6C^{lo} cells [64, 65]. Moreover, the converting of Ly6C^{hi} monocytes into Ly6C^{lo} monocytes has been reported in hepatic injury, which contributes to optimal tissue repair. Ly6C^{hi} monocytes expressed TNF and IL-1β that were only recruited in the first days of tissue injury [66]. Similar monocyte conversion occurs in skeletal muscle injury. Ly6C^{hi} monocytes phagocytoses debris and then rapidly convert to Ly6^{lo} monocytes exhibiting features of anti-inflammation, contributing to myogenesis and fiber growth after inflammation was resolved [67]. These findings provide new insight of the plasticity of monocytes and demonstrate that characteristics of monocytes can be educated and shaped depending on the tissue microenvironment.

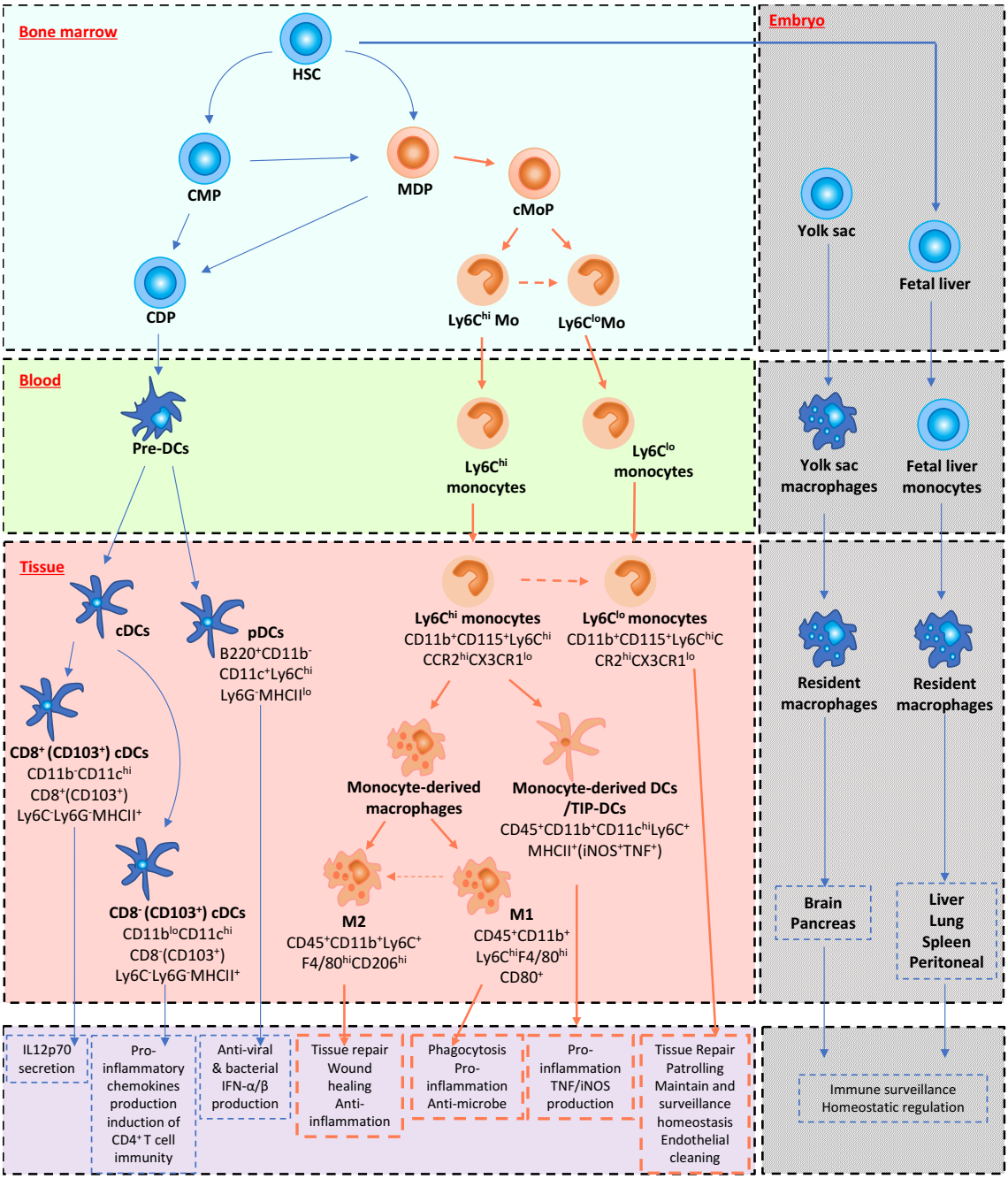


Figure 1.4 Origin and differentiation of macrophages and immune cells of myeloid origin

Monocytes are continuously generated from HSCs in the bone marrow. HSC firstly give rise to common myeloid precursors. Subsequently, they establish MDP and CDP. CDP was shown to generate conventional DCs (cDCs) and plasmacytoid DCs (pDCs) except monocytes. pDCs accumulate in blood and lymphoid tissue and are the main producers of type I interferon, which plays a key role in anti-viral and anti-bacterial immunity. Conversely, cDCs mainly locate in non-lymphoid tissue, where they continually acquire tissue and blood antigens in the steady circumstance.

MDP give rise to a cMoP, which are the direct progenitors of Ly6C cells. Ly6C^{hi} monocytes are recognized as classical monocytes, recruited to the site of inflammation as a precursor of functional cells, such as Mo-M, Mo-DCs and TNF-iNOS producing DCs. Ly6C^{hi} monocytes can differentiate into a Ly6C^{lo} population in the circulation. Ly6C^{lo} monocytes patrol the luminal side of endothelium of the small blood vessels, where they perform immune surveillance. During inflammation, they are recruited and secrete anti-inflammatory cytokines and contribute to tissue repair.

Hematopoiesis in the fetal liver generates macrophages which become the main sources of most tissue macrophages in adults. These cells are contributing to immune surveillance and homeostatic regulation.

Very primitive macrophages are generated from yolk sac.

1.2.2 Monocyte markers

Unfortunately, there is currently no unified nomenclature for distinguishing between monocytes, macrophages, and DCs since their characteristics are often overlapping. The notion of ontogeny of the cells has been totally changed, and how to define the cell types is a challenge. Although researchers still use common marker molecules such as CD45, CD11b/c, F4/80, Ly6C/G, C-C chemokine receptor type 2 (CCR2) and CX3C chemokine receptor 1 (CX3CR1), they are not sufficiently discriminating to allow a clean separation of different cell subsets if these markers are used alone. Therefore, it is important to understand the ontogeny of the cells as well as the markers that help researchers to choose the appropriate marker combination to identify cells with different phenotypical characteristics. Here, I will introduce the most frequently used marker molecules.

CD45 is a receptor-linked tyrosine phosphatase [68], and expressed on all leucocytes, but it is differentially expressed in various lineages providing an additional means to distinguish subpopulations. CD45 is expressed at highest levels on T lymphocytes and NK cells, while in B cells and monocytes the intensity of its expression is dependent on the degree of B cell and

monocytes maturation. In contrast, erythroid cells are $CD45^{dim}$ and lose CD45 expression during the maturation process [69, 70].

A frequently analyzed marker in the investigation of an inflammatory infiltrate is the expression of the heterodimeric adhesion molecules $\alpha_m\beta_2$ (CD11b-CD18) and $\alpha_x\beta_2$ (CD11c-CD18). Both molecules are broadly aligned with either monocytes/macrophages or DCs, respectively [71]. Importantly, while CD11b is normally expressed on myeloid lineage cells, it is also present on some lymphoid cells such as natural killer (NK) cells [72]. It is therefore, not conclusive if used alone. On a similar note, the integrin CD11c is commonly recognized as a marker of DC, including conventional DC, inflammatory monocytic DC as well as plasmacytoid DCs, but can also be present on macrophages and monocytes *in vivo* [73].

M-CSFR is encoded by the c-*fms* proto-oncogene. The receptor is expressed on many cell types of the MPS [74] and has been studied extensively using MacGreen [75] and MacBlue transgenic reporter mice [76]. It has been shown that the receptor is on a majority of blood monocytes but is lost on many tissue resident macrophages [76]. Therefore, the reliance of cells of the myeloid linage on this receptor for their development and survival was tested using an anti-CSF-1 R antibody to block the receptor. Interestingly, treated mice show only a very specific reduction of subpopulations of blood monocytes after three weeks of treatment ($F4/80^{hi}CSF1\text{-}R^{hi}$, $GR1^-$ but not $F4/80^{int}CSF1\text{-}R^{int}$, $GR1^+$) and a subset of tissue macrophages (liver, gut, and kidney but not brain, lung, ovary and uterus) [77].

Macrophages are often distinguished from DCs and monocytes cells by differential expression of the surface marker F4/80, which is encoded by an EGF-like module containing a mucin-like hormone receptor-like sequence 1 [78] and constitutively expressed on many cells of the

myeloid lineage, such as tissue-resident macrophages (F4/80^{hi}) [79]. Recent work has shown that hematopoietic stem cell-derived CD11b^{hi} monocytes and macrophages depend on the transcription factor Myb while earlier yolk-sac-derived macrophages are independent of Myb [80]. This early generation of macrophages can be found in liver (Kupffer cells), skin (some Langerhans cells) and brain (microglia) and has been described as F4/80^{hi} [80]. These results demonstrate that F4/80 can be used in some populations as marker of the origin of myeloid cells.

Two molecules of the Ly-6 family of molecules, Ly6C and Ly6G, are constitutively expressed on a proportion of myeloid cells. Both molecules share one epitope that is recognized by the mAb RB6-8C5. This mAb was originally described as binding to a surface antigen present on mature granulocytes (neutrophils and eosinophils) [81]. Subsequently, it could be demonstrated that the epitope recognized by RB6-8C5 was also present to a lower extent on monocytes [82] and the generation of other antibodies such as ER-MP20 allowed the separation of the two antigens and the specific staining of Ly6C^{+} cells [83]. Additionally, the use of new depleting Ly6G-specific monoclonal antibodies such as 1A8 made it possible to revisit older depletion experiments and arrive at better results [84]. Meanwhile, Ly6C has been established as important inflammatory marker on monocytes [85, 86] and can be used together with Ly6G to determine relative proportion of granulocytes and monocytes or macrophages.

Two members of the chemokine receptor family, CCR2 [87] and CX_3CR1 [88], have been identified as mutually regulated marker molecules for different populations of monocytes [88]. Cells that are highly positive for CX_3CR1 were shown to patrol vessels and healthy tissues and were able to enter the tissue rapidly when recruited [60]. In contrast, the receptor CCR2 marked circulating monocytes and was of particular interest because it is crucial for the egress from

the bone marrow during inflammation and therefore marking inflammatory, bone marrow-derived monocytes [87, 89]. Interestingly, the level of Ly6C expression on the cell surface is associated with either the CX_3CR1 receptor, ($CCR2^{lo}CX_3CR1^{hi}Ly6C^{lo}$), or the CCR2 expression ($CCR2^{hi}CX_3CR1^{lo}Ly6C^{hi}$) and can be used in combination to differentiate between populations of monocytes.

1.2.3 The roles of monocytes during leishmaniasis

Besides the cellular immune response, control of *Leishmania* infection in the mammalian host is primarily mediated by the innate cellular immune response. Monocytes have been demonstrated to be a very important cell population contributing to the initial innate immune response and to priming the adaptive immune response during *Leishmania* infection [90]. Monocytes can be rapidly recruited to the site of infection where they give rise to macrophages or DCs in response to different environmental signals.

In the mouse model of visceral leishmaniasis, the role of monocytes as a distinct subset has not been investigate directly, but there are observations that point to an involvement. It has been demonstrated that depletion of $Ly6C^+$ monocyte increased the parasite burden [91]. Moreover, monocytes contribute to granulomas formation, reducing parasite load in the liver [22], and depletion of monocytes delayed the maturation of hepatic granulomas [92]. Additionally, monocyte-derived macrophages exhibiting a M2 macrophage phenotype are also regarded to be characteristic for Post kala-azar dermal leishmaniasis, a chronic dermatosis caused by human leishmaniasis [93].

In *L. major* infected mice, monocytes are rapidly recruited to the dermis and differentiate into dermal Mo-DCs. This population then migrate to the draining lymph nodes, present parasitic

antigen to T cells and prime the protective Th1 immune response to infection. Although monocyte recruitment and differentiation are also found in the draining lymph node, monocyte-derived DCs from draining lymph node are less efficient in promoting IL-12 production and IFN-γ production by $CD4^+$ and $CD8^+$ T cells [94]. In addition, a similar Mo-DCs population is found in the footpads as well as draining lymph nodes in the B6.WT mice lacking of TNF gene ($B6.TNF^{-/-}$) mice during *L. major* infection. However, this population exhibit the phenotype more like M2 macrophage, with less iNOS expression and harboring a large number of parasites due to the absence of TNF [95]. Therefore, it is argued that Mo-DCs can be reservoirs of *Leishmania* parasites. Their ability to successfully eradicate parasites is dependent on their degree of maturation and activation state [96-98]. Immature DCs internalize antigen by phagocytosis [99, 100], and they migrate to lymph nodes, where they become mature and capable of stimulating naïve T cells with the help of up-regulated chemokine receptors on their surface. Impairment of this fundamental process leads to disease progression. It was reported that the virulence-associated antigen of *L. donovani* promastigotes inhibited Mo-DCs maturation by decreasing CD86 expression [101]. Moreover, maturation of Mo-DCs is accompanied by a loss of adhesion to surrounding tissue [102]. *L. donovani* promastigotes elevated integrin CD11b and CD51 expression on Mo-DCs to prevent their detachment and migration to lymphoid organs [101]. Mice exhibited enlarged lesions and increased parasitic burden due to defective monocyte maturation during *L. major* infection. An increased number of myeloid-derived $CD11b^+Gr1^+$ cells were found harboring a large amount of *L. major* parasites, and exhibiting an immature phenotype, with lower expression of F4/80 expression and increased IL-10 secretion, suppressing the inflammatory monocyte function [103, 104].

The importance of the role of inflammatory monocytes is also demonstrated in cutaneous infection models using *Leishmania* strains other than *L. major*. After infection of with *L.*

mexicana, the C57BL/6 mice developed non-healing cutaneous lesions [105]. Infected mice showed a reduced recruitment of $CD11b^{+}CD11c^{-}Ly6C^{+}$ inflammatory monocytes to the skin and the local lymph nodes. Consequently, they failed to control the lesion and a chronic infection developed [106]. Adoptive transfer of Mo-DCs helped to overcome the infection [106]. In contrast to *L. mexicana*, a cutaneous infection with *L. braziliensis* caused small, self-healing lesions in both C57BL/6 and BALB/c mice [107]. It was demonstrated that a potential mechanism supporting this resistance in BALB/c mice could be the absence of a strong IL-4 response causing a Th1 type response and consequently, a strengthening of the pro-inflammatory aspect of innate immunity [108]. A more detailed investigation of the *L. braziliensis* pathology emphasized the role of iNOS and TNF in the protective response to this infection [109] and in a recent study the predominant protective myeloid population was identified as $CD11b^{+}CD11c^{-}Ly6C^{+}$ inflammatory DC [110].

Monocyte derived macrophages have been comparatively less studied than Mo-DCs during leishmaniasis. Similar to normal macrophages, Mo-M have also been reported have distinctive activation status, with recruited inflammatory macrophages and Mo-M displaying M2 macrophage features [111]. Although recruited inflammatory macrophages can produce pro-inflammatory cytokines and induce inflammatory responses, it was found they were the cells that amastigotes chose to infect [112]. These cells show a delay in their activation of their antileishmanial activities due to the lower temperature of the skin (34-36 ºC) [113] and the cytokines induced during immune response [114]. Moreover, parasites persist in the tissue that induce continuous influx of inflammatory macrophages, facilitating parasite replication by providing them the constant shelters [115]. Similarly, Mo-M from *Leishmania* infected patients were demonstrated to produce less NO, and this population displayed features of M2 polarization including increased expression of classical M2 markers CD206 and Arg-1 [93].

Therefore, monocyte differentiation directly affects the outcome of *Leishmania* infection. It can be envisaged that the strategy of determination and reorientation of this process could be a therapeutic modality to conquer a disease like leishmaniasis.

1.3 Tumor necrosis factor

Tumor necrosis factor was first discovered by Carswell in 1975, who found there was an endotoxin-inducible molecule that caused necrosis of tumors [116]. In 1985 human TNF was purified [117] and it was demonstrated that it synergized with IFN-γ to induce the production of NO [118]. TNF is a 26KDa transmembrane protein produced mainly by immune cells such as macrophages, DCs and T cells and is expressed on the cell surface. It can be cleaved by a specific matrix metalloproteinase, resulting in soluble TNF. Response to TNF is transferred via two distinct receptors: the pro-inflammatory TNF receptor-1 (TNFR1, also named TNFp55) and the homeostatic TNF receptor-2 (TNFR2, termed TNFp75) [119]. TNFR1 is expressed ubiquitously, and predominately binds soluble TNF. While, TNFR2 is restricted and expressed on immune cells, neurons and endothelial cells, and preferentially binds pro-TNF, which acts in a complementary or ligand-delivery role to TNFR1 [120]. TNFR1 is normally associated with tissue injury involved in the pro-inflammatory and the programmed cell death pathways, whereas TNFR2 is often recognized as a homeostatic receptor, mediating local homeostatic effects, such as cell survival and tissue regeneration [121]. Of note, like TNF, TNFR can also be cleaved from the cell surface by TNF-converting enzymes generating a soluble TNFR, which acts as a regulator of the inflammatory response by inhibiting TNF activity [122].

Normally TNF cannot be detected in the plasma of healthy people, but it accumulates when inflammation occurs [123]. Rheumatoid arthritis is a chronic inflammatory disease, characterized by an excess of TNF production, that causes persistent synovitis and systemic

inflammation and damages the joints in RA patients. The levels of TNF in patients' serum correlated with the severity of RA, as well as being a key marker of RA activity [124]. Thus, either neutralizing TNF or blocking TNF and TNFR binding is beneficial in RA treatment especially in cases refractory to conventional therapies. Infliximab and adalimumab (monoclonal antibodies), and etanercept (a TNF receptor fusion protein) are three agents currently used clinically that have dramatically improved the outcomes of RA (Table 1). However, opportunistic infection as a side effect is a safety concern in patients on anti-TNF therapy and a number of case reports have stated that clinical use of anti-TNF therapy caused an increased risk of infection, including leishmaniasis [125-128]. Patients have been found with cutaneous and mucocutaneous leishmaniasis infection after receiving therapy with adalimumab [129]. Also, an RA patient was reported having a relapse of the latent leishmaniasis after receiving treatment with adalimumab [130]. Therefore, awareness of the therapeutic potential and associated adverse events is necessary for maximizing therapeutic benefits while minimizing adverse effects from anti-TNF treatments.

Table 1. Frequency of use different anti-TNF agents

Generic name	Trade name	Type of biologic	Dosage
Etanercept	Enbrel	TNF receptor fusion protein with Fc region of IgG	50 mg S.C. weekly
Infliximab	Remicade	Chimeric murine/humananti-TNF monoclonal antibody	3 mg/kg I.V. every 8 week
Adalimumab	Humira	Recombinant human anti-TNF monoclonal antibody	40 mg S.C. every 2 week
Certolizumab	Cimzia	Human anti-TNF monoclonal antibody	400 mg S.C. at week 0, 2, 4 then every 4 weeks
Golimumab	Simponi	Fully human anti-TNF monoclonal antibody	50 mg S.C. monthly

S.C. subcutaneous; I.V. intravenous

The production of NO is the key defence mechanism against *Leishmania* infection. In Th1 cytokines stimulated-macrophages, iNOS expression is upregulated and catabolizes the substrate L-arginine towards NO generation, contributing to the parasite killing and elimination. TNF is a very important cytokine involved in this process. It has been demonstrated that TNF synergizes with IFN-γ to induce robust increases of NO synthase activity, and impairment of TNF signaling reduces activity of macrophages as well as fails to produce NO in response to IFN-γ [131]. Moreover, resistant mice lacking the TNF gene have been found to rapidly succumbs to *L. major* infection. Although their Th1 immune response was not impaired, as there were large amounts of IFN-γ, these mice still displayed non-healing skin lesions and progressive infection of the internal organs. The outcome of the infection was fatal even when only infected with 3000 promastigotes [132, 133], indicating the indispensable role of TNF in leishmaniacial response. Recently, Schleicher *et al* found that upregulated Arg-1 accounts for the fatal outcome of B6.TNF$^{-/-}$ mice [134]. Arginase-1 catalyses the hydrolysis of L-arginine to urea and L-ornithine. The production of ornithine contributes to the generation of polyamine, which is essential for parasite replication. Since Arg-1 and iNOS use L-arginine as the same substrate, the competition of L-arginine between iNOS and Arg-1 is critical for determination of parasite infection. TNF inhibits IL-4-induced Arg-1 expression by decreasing histone acetylation, resulting in enhanced production of NO by iNOS in macrophage and DCs [134]. While, absence of TNF leads to hyperexpression of Arg-1 that impairs the production of leishmaniacidal NO [134], demonstrating the importance of TNF in iNOS/NO-mediated anti-leishmaniasis response.

In addition, TNF has been reported to be involved in mediating the monocyte/macrophage differentiation process. It has been shown that TNF counteracts IL-4-driven activation of M2 macrophage, thereby inhibiting Arg-1-induced parasite replication [134]. In the absence of

TNF monocytes differentiate into a Ly-6C^{lo} population with M2 macrophage phenotype that are permissive for *L. major* infection [95], indicating the key role of TNF in maintaining normal monocyte differentiation.

1.4 Interleukin 6 and its signaling

As a multi-functional, pleiotropic cytokine, IL-6 was originally considered to promote the activation and expansion of T cells [135] and play a role as B cell differentiation factor that induced plasma cell differentiation and promoted the production of immunoglobulin G [136]. IL-6 is produced by various cell types including hematopoietic (T-cells, B-cells, monocytes, and macrophages) and non-hematopoietic cells (fibroblasts, keratinocytes, endothelial cells, mesangial cells, adipocytes and some tumor cells) [137]. Therefore, it is able to influence numerous biological processes by formation of a complex with its two receptors: type I transmembrane glycoprotein IL-6R and type I transmembrane signal transducer protein gp130. On the target cells, IL-6 first bind to the non-signaling, membrane-bound receptor IL-6R (mIL-6R), which triggers homodimerization of gp130 binding [138]. The dimerization of IL-6/mIL-6 gp130 then activate Janus activated kinase (JAK) and induces JAK to auto-phosphorylate. Subsequently, this activates signal transducer and activator of transcription (STAT), ERK and phosphatidylinositol 3-kinase signaling pathways [139]. Although IL-6R is important in signal transduction, mIL-6R-mediated responses are very limited. Unlike the ubiquitously distributed gp130, mIL-6R only express on hepatocytes, some epithelial cells and some leukocytes [140]. Therefore, mIL-6R-mediated response is restricted to cells that express mIL-6R. Another mode of action other than classical signaling is IL-6 trans-signaling. A soluble form of IL-6R (sIL-6R) is generated either by proteolytic cleavage or alternative splicing and released from the cell surface, and then binds IL-6 to form an agonistic complex that directly activates cells

through gp130. It expands the active response from IL-6R-positive cells to all gp130-expressing cells and extends IL-6 bioactivity in the body to nearly all cells [141].

IL-6 is induced together with other pro-inflammatory cytokines, such as TNF, that are involved in inducing pro-inflammatory responses. Absence of IL-6 results in higher mortality by weakened immune defence in the bacterial infection [142]. However, accumulating evidence suggest an anti-inflammatory role of IL-6 in a variety of pathogen/physiological conditions. IL-$6^{-/-}$ mice shows increased levels of pro-inflammatory cytokines such as TNF and macrophage inflammatory protein 2, and administration of recombinant IL-6 to these mice abolished differences, supporting the anti-inflammatory role of IL-6 [143]. Hepatocytes are one of the major cell populations expressing mIL-6R. Specific blocking of IL-6R on hepatocytes increased systemic inflammation and reduced glucose tolerance[144], and mice lacking of IL-6 showed impairment of liver regeneration [145]. Furthermore, IL-6 has been proved to promote alternative macrophage activation that limits obesity-associated insulin resistance and edotoxemia [146], and also restrains the neuro-inflammation [147].

1.5 Scope of study

The critical role of TNF during *L. major* infection has been demonstrated by a series experiments using TNF neutralizing reagents or TNF deficient mice. In the absence of TNF, progressive infection of internal organs was found during *L. major* infection, resulting in uncontrolled parasite replication and aberrant immune responses leading directly or indirectly to the death of the host. It is poorly understood how *L. major* infections occur in solid tissue organs and what the underlying mechanism is that mediates the fatal outcome. Such information is necessary not only to understand the immune response in the absence of TNF

during leishmaniasis, but also to help researcher to envisage potential strategies to fight this disease.

Previously, the evidence from our laboratory showed loss of TNF facilitates *L. major* infection by skewing the macrophage expression profile. A monocytic population displaying an alternative macrophage phenotype were found in the footpads and popliteal lymph nodes of B6.TNF$^{-/-}$ mice. These monocytes harbored a large number of parasites and displayed a low iNOS expression [95] but a high Arg-1 expression in the absence of TNF [134]. Such a finding is consistent with the idea that TNF has a key role in the NO production and monocyte/macrophage differentiation process, indicating that these abnormalities may not be restricted to these specific tissues.

Therefore, it is hypothesized that abnormal monocyte/macrophage differentiation and imbalanced iNOS/Arg-1 production also occurs in the internal organs liver and spleen, which account for visceral infection and a fatal outcome during *L. major* infection of B6.TNF$^{-/-}$ mice.

To investigate this hypothesis, we aimed to:

1) To confirm liver and spleen infection during cutaneous leishmaniasis in the absence of TNF.
2) To explore the reason leads to the visceral liver infection in the absence of TNF during disease progression.
3) Identify the potential molecules and understand how does it interact with TNF that are involved in the disease progression.
4) Examine and explain the potential mechanism that induces spleen infection Identify the potential molecules interacts with TNF that are involved in the disease progression.

Chapter 2.
General materials and methods

2.1 Mouse strains

Control B6.WT mice and BALB/c mice were purchased from The Jackson Laboratories (Bar Harbor, USA) and imported directly to the University of Tasmania Cambridge Farm Facility. B6.TNF$^{-/-}$ mice [148] were bred at the University of Tasmania Cambridge Farm Facility. All the mice were kept at this location before being transferred to specific pathogen-free conditions at the animal facilities of the Menzies Institute for Medical Research, Hobart, Australia. Mice were used for the experiments at 6-12 weeks of ages. Infection experiments were performed with sex- and age-matched B6.WT and B6.TNF$^{-/-}$ mice. BALB/c mice were infected at the same age as B6.WT and B6.TNF$^{-/-}$ mice and used for parasite passage and disease positive control. All animal procedures were approved by the animal care and experiments were approved by the Animal Ethics Committee of the University of Tasmania, Hobart, Australia (Animal Ethics Number: A13934 & A13935), in accordance with the Australian Code of Practices and Guidelines, set out by National Health and Medical Research Council.

2.2 *Leishmania major* culture and *in vivo* infection

The virulent *L. major* BNI (MHOM/IL/81/FE/BNI) was kindly provided from Prof. Christian Bogdan (Institute of Microbiology, Erlangen, Germany). The infectivity of the parasites was maintained by passaging through the tissue of susceptible BALB/c mice as described [132]. Prior to infection, the parasites were cultured *in vitro* in Novy-MacNeal-Nicolle blood agar slants in RPMI 1640 containing 10% rabbit serum (Applied biological products management-Australia, South Australia, Australia), penicillin/streptomycin, nonessential amino acids, and 10mM HEPES, all supplied by Gibco (Life Technologies, VIC, Australia) [95, 149]. Mice were injected bilaterally into the skin of the hind footpads with $3x10^6$ stationary-phase *L. major* promastigotes. Clinical monitoring of the infection was performed by measuring the footpad

thickness with a metric caliper [132] and by recording the body weight of mice at different time points.

2.3 Sample collection

Mouse whole blood samples were collected into regular 1.5ml Eppendorf tubes by cheek-bleeding before mice were sacrificed at different time points. The tubes were held in an upright position at room temperature for 45-60 minutes (mins) to allow clotting and then centrifuged for 15 mins at x1500g, room temperature. Mouse serum was recovered by pipette and transferred into a fresh tube and stored in -80°C for further use.

Mice were sacrificed by CO_2 and then perfused manually. A lateral incision was made through the integument and abdominal wall to expose the pleural cavity. Cuts through the rib cage up to the collarbones on the both sides allowed the sternum to be lifted away to expose the heart. A 21-gauge needle was introduced into the ascending aorta, and an incision was made to the right atrium using iris scissors. Finally, perfusion of the mice was performed using with 30ml PBS and 10mM ethylenediaminetetraacetic acid (EDTA) slowly via the 21-gauge needle. Livers and spleens were then removed and stored as different experiments required.

2.4 Limiting dilution assay of parasite burden

Limiting dilution experiments were performed to determine the parasite burden in the infected livers and spleens. Single cell suspensions were prepared in supplemented Schneider's media and serial dilutions (3-fold for liver and 10-fold for spleen) were pipetted across a 96-well plate with 12 replicates in an end-point titration. The plates were incubated for 10-14 days at 27°C before the number of *Leishmania*-positive wells was determined using both a light microscope

Olympus CKX31 (Australia) and Spectra Max/M2 Microplate reader (Molecular Device, CA, USA). To calculate the parasite burden, a generalized Pearson *chi*-square test was performed by L-Calc (version 1.1, STEMCELL Technologies, BC, Canada).

2.5 Liver mononuclear cell isolation

Isolation of liver mononuclear cells was performed by cutting the organ into small pieces and then digesting them in Hank's balanced salt solution containing Collagenase II (100U/ml, Gibco, Life Technologies) and DNase I (1U/µl, Sigma-Aldrich, NSW, Australia), for 30 mins at 37°C shaking at 200 rpm. The suspension was filtered through a 100µm strainer (Thermo Fisher Scientific, VIC, Australia) to remove the major tissue fragments. Following centrifugation at 80×g for 3 mins at 4°C, cells were re-suspended and loaded onto Histopaque 1083 (Sigma-Aldrich) followed by centrifugation at 1400×g for 20 mins at 4°C. The cells at the interface between the plasma and Histopaque 1083 were harvested. Harvested cells were washed by resuspension in 10ml phosphate buffered saline (PBS) pH 7.2 followed by centrifugation at 600×g for 10 mins at 4°C. The cell pellet was re-suspended in PBS (1ml) and used for determination of the cell number and further experiments.

2.6 Spleen mononuclear cell isolation

Spleens were harvested and cells were isolated by cutting the organ into small pieces and then digesting them in hank's balanced salt solution containing Collagenase II (Gibco, Life Technologies) and DNase I (Sigma-Aldrich) 30 mins at 37°C shaking at 200rpm. The suspension was filtered through 100µm strainer (Thermo Fisher Scientific, VIC, Australia) to remove the major tissue fragments. After 1400×g 5 mins centrifugation at 4°C, cells were depleted of erythrocytes using red blood cells lysis buffer for 5 mins on ice, and the process

stopped by the addition of PBS to 15ml. The cells were collected by centrifugation at 600×g for 5 mins at 4° C, and the wash step repeated twice. Finally, the cell pellet was re-suspended in PBS (1ml) and used for determination of the cell number and further experiments.

2.7 Flow Cytometry, and Cell Sorting

Multi-colour flow cytometry was performed on single cell mononuclear suspensions from liver and spleen. Liver cells were stained first for surface marker expression with rat anti-mouse CD45 (Biotinylated; 30-F11; BD Pharmingen, NSW, Australia), rat anti-mouse Ly6C (FITC; clone AL-21; BioLegend, West Australia, Australia), rat anti-mouse F4/80 (APC-Cy7; clone BM8; eBioscience, VIC, Australia), and rat anti-mouse CD11b (PerCP-Cy5.5; clone M1/70; BD Pharmingen). The cells were then fixed with FoxP3 Fix/Perm buffer and permeabilized with FoxP3 Perm buffer (BioLegend) according to the manufacturer's protocol. Intracellular proteins were targeted with rat anti-mouse CD206 (PE; clone C068C2; BioLegend), rat anti-mouse IL-10 (PE; clone JES5-16E3; BD Pharmingen), rat anti-mouse IL-4 (PE; clone 11B11; BD Pharmingen), rat anti-mouse IL-6 (PE; clone MP5-20F3; BD Pharmingen), purified anti-*L. major* (clone V121) and mouse anti-mouse Arginase1 (PE; clone polyclone; R&D Systems, VIC, Australia). Streptavidin conjugated to V500 (BD Pharmingen) was used as a secondary stain. Cells were acquired on a BD FACS Canto II flow cytometer (Becton Dickinson, NJ, USA) using BD FACS Diva version 6.1.3 and analysis were performed with FlowJo software version 10.1 (Tree Star, Inc.). For flow cytometric cell sorting, two population $CD45^{+}F4/80^{+}CD11b^{+}Ly6C^{lo}$ and $CD45^{+}F4/80^{+}CD11b^{+}Ly6C^{hi}$ were sorted by MoFloAstrios (Beckman Coulter, CA, USA).

Staining of spleen cells was performed according to three different staining panels listed in the Table 2. In order to identify sub-populations of DCs in spleen, an additional staining panel was required. Spleen cells firstly were stained with Ly6G (Brilliant violet 421; clone IA8; BD Pharmingen or eBioscience), NK1.1(FITC; clone PK136; BD Pharmingen), TCR (FITC; clone 145-2C11; BD Pharmingen) and CD19 (FITC; clone ID3; BD Pharmingen or eBioscience) to exclude neutrophils, NK cells, T cells and B cells, respectively. After sorting, $Ly6G^{-}NK1.1^{-}CD3^{-}CD19^{-}$ cells were further stained with different cell surface markers listed in the Table 2 Panel 3.

Table 2. Different flow cytometry antibodies applied in spleen cells

	Host	Target	Fluorochrome	Clone	Supplier
Panel 1	Rat	CCR2	-	MC-21	M. Mack, Regensburg
	Donkey	Rat IgG	Alexa Fluor 647	-	Jackson Immunoresearch
	Rat	CD11b	Percp-Cy5.5	M1/70	BD pharmingen
	American hamster	CD11c	PE-Cy7	HL3	BD pharmingen
	Rat	Ly6C	FITC	AL-21	BioLegend
	Rat	Ly6G	Brilliant violet 421	RB6-8C5	BioLegend
	Rat	F4/80	APC-Cy7	BM8	eBioscience
Panel 2	Rat	CD11b	Biotin	M1/70	BD pharmingen
	Rat	CD19	FITC	ID3	BD pharmingen
	American hamster	CD3	APC	145-2C11	BD pharmingen
	Rat	CD4	Percp-Cy5.5	RM4-5	BD pharmingen
	Rat	CD8α	Pacific Blue	53-6.7	BD pharmingen
	-	Streptavidin	V500	-	BD Pharmingen
Panel 3	Rat	B220	FITC	RA3-6B2	BD pharmingen

	Rat	MHCII	PE	AF6-120.1	BD pharmingen
	Rat	CD11b	Percp-Cy5.5	M1/70	BD pharmingen
	American hamster	CD11c	PE-Cy7	HL3	BD pharmingen
	Rat	CD8α	Pacific Blue	53-6.7	BD pharmingen
	Rat	Ly6C	APC	HK1.4	eBiosciences

Stimulated bone marrow-derived cells were stained to distinguish DCs and M2 macrophage population using with CD11b, F4/80, CD206 and M-CSFR, separately, as different experiments required (Table 3).

Table 3. Flow cytometry antibodies applied in bone marrow derived-cells

	Host	**Target**	**Fluorochrome**	**Clone**	**Supplier**
Panel 1	Rat	F4/80	APC-Cy7	BM8	eBioscience
	Donkey	M-CSFR	APC	AFS98	Biolegend
	Rat	CD11b	Percp-Cy5.5	M1/70	BD pharmingen
	Rat	CD206	PE	C068C2	Biolegend

2.8 Cytometric bead arrays assay

Cytometric bead arrays (CBA) assay was performed on the serum collected from the peripheral blood of infected mice. Mouse inflammation kit (BD Biosciences, NSW, Australia) was used to determine the cytokine expression of IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12 p70, as per the manufacturer's instructions were essentially followed. Samples were acquired on a BD FACSCanto II using FACSDiva 6.1 software and analyzed with FCAP Array version 1.0 software (BD Biosciences, NSW, Australia).

2.9 Immunohistochemistry

Liver and spleen tissue specimens were fixed in formalin and embedded in paraffin. Histological sections of 4μm in thickness were stained with hematoxylin and eosin (H&E) using standard protocol. The histopathological changes before and after *L. major* infection were observed and photographed using an optical microscope (Leica DM2500, North Ryde, Australia).

For immunohistochemical staining, tissue sections were de-paraffinized in xylene and rehydrated using a graded series of ethanol/water solutions. These sections were retrieved with Proteinase K (Dako, VIC, Australia) for 3 mins at room temperature before being stained. Endogenous peroxidase activity was quenched by treatment with 3% H_2O_2 in distilled water for 10 mins. The sections were blocked in protein block (X0909, Dako) for 10 mins at room temperature to prevent non-specific binding and then incubated with primary antibodies to anti-CD68 (ab31630, Abcam, VIC, Australia) for 1 hour at 37°C. Binding of the primary antibody was detected using visualized using the envision system (Dako) according to the manufacturer's protocol, and diaminobenzidine (DAB) was used as the chromogen. The nuclei were lightly counterstained with haematoxylin solution. A negative control was prepared using the same staining procedure but was not incubated with the above-mentioned primary antibodies. Images were obtained using an Olympus BX53 microscope, and semi-quantitative analysis was conducted using Image-Pro Plus software (Media Cybernetics, USA).

Flow cytometrically sorted cells were centrifuged on slides in a Cytospin^{TM} 4 Cytocentrifuge (Thermo Fisher, Wayland, USA) at 500×g for 10 mins. Subsequently, the slides were air-dried for further use. For Diff-Quik staining, air-dried cytospin slides were incubated for 1min in

Fixation Solution of a Diff-Quik kit [150]. Subsequently, incubated for 1min in staining solution I followed by 1min in staining solution II. The slides were rinsed in tap water, air-dried and used for further microscopical analyses. For immunofluorescence staining, cells were fixed with formalin for 30 mins and stored for further investigation.

2.10 Immunofluorescence

Liver tissue samples were dissected and rapidly frozen in Tissue-Tek optimal cutting temperature medium (VWR, Queensland, Australia) in liquid nitrogen vapor and stored at -80°C. Sections (8μm) were cut using a cryotome (Thermo Fisher), air-dried and fixed in acetone at -20°C. Prior to staining, sections were rehydrated in PBS/1% BSA for 60 mins. The primary antibodies and the second labelling reagents are described in Table 4. Sections were mounted with polyvinyl alcohol mounting media with DABCO (Sigma-Aldrich) to prevent fading. Polyclonal IgG antibodies specific for *L. major* (clone V121, MHOM/IL/67/Jericho II) were purified from an anti-*Leishmania* rabbit serum (a generous gift from Emanuela Handman) using Protein G-Sepharose 4B (Invitrogen), followed by labelling with Cy5 mono-reactive dye (Amersham Biosciences, Buckinghamshire, England) as described by the manufacturer. Immunofluorescence images were visualised and obtained using UltraView Spinning disc confocal microscope with Velocity Software (Perkin Elmer, Massachusetts, USA). The objective magnification is 40×. The laser line used here are 405nm, 488nm, 561nm and 640nm, and the corresponding filters are DAPI (445/60), Alexa (488, 485/60), Alexa (546, 525/50) and Alexa (647, 705/90). All the images were processed using ImageJ version 1.50i (ImageJ, U.S.A).

Table 4. Primary and secondary antibodies used in fluorescence and confocal microscopy

	Host	Target	Fluorochrome	Clone	Supplier
Primary Antibodies	Rabbit	*L. major*	-	V121	M. Mack, Regensburg
	Rat	CD11b	Biotin	M1/70	BD pharmingen
	Mouse	iNOS	-	6/iNOS/NOS Type II	BD Biosciences
	Rat	CD206	-	MR5D3	BD pharmingen
	Rat	IL-6	Biotin	MP5-32C11	BioLegend
Secondary Reagents	Rat	Streptavidin	Alexa Fluor 546	-	Life Technologies
	Donkey	Rat IgG	Alexa Fluor 647	-	Life Technologies
	Goat	Rabbit IgG	Alexa Fluor 488	-	Life Technologies
	Goat	Mouse IgG	Alexa Fluor 647	-	Life Technologies

2.11 Bone marrow-derived macrophages and dendritic cell *in vitro* culture

Bone marrow cells were flushed from femur and tibia of uninfected B6.WT and B6.TNF$^{-/-}$ mice and cultured in RPMI1640 media (Gibco, Life Technologies) either with supernatant containing M-CSF obtained from L929 cells or supernatant containing previously titrated granulocyte macrophage colony stimulating factor (GM-CSF) harvested from X63-AG8 cells from day 7 to day 10 of culture. Recombinant mouse IFN-γ (20ng/ml, Peprotech, VIC, Australia) and IL-4 (10ng/ml, Peprotech) were added into the medium for differentiating into M1 and M2 macrophage, respectively. Then, recombinant mouse IL-6 (10ng/ml, Peprotech), and TNF (10ng/ml and 50ng/ml, Peprotech) were added as the stimulant and stimulants. To assess nitric oxide and cytokine production, cells were rinsed with PBS and activated with LPS (100ng/ml, Peprotech) for an additional 24 hours following the cytokine stimulation.

2.12 Quantitative real-time polymerase chain reaction (PCR)

RNA was extracted from sorted liver cells and cultured cells by using Tri-Reagent (Sigma-Aldrich) or RNA isolation mini kit (Bioline, Alexandria, Australia) according to the manufacturer's instructions. RNA was stored in RNase-free water at -80°C. The QuantiTect Reverse Transcription Kit (Qiagen, Melbourne, Australia) was used to reverse-transcribe up to 1000ng total RNA. cDNA (2µl) was amplified by Quantitative real-time PCR on the Rotor-Gene Q qPCR instrument (Qiagen, Melbourne, Australia) with 10µl reactions using the SensiFAST^{TM} SYBR No-Rox Kit (Bioline, Alexandria, Australia). The appropriate oligonucleotide primers were listed in Table 5, and the reaction was performed in a predetermined condition. Samples were heated at 95°C for 3 mins and amplified with 40× cycles of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 30 seconds. Reactions were performed in duplicate and gene expression levels were normalized to β-actin. Relative gene expression between samples was calculated using the $2^{-\Delta\Delta Ct}$ calculation method [151].

Table 5. Primers used for quantitative PCR characterising monocytes from liver and bone marrow derived cells

Gene	Forward Primer	Reverse primer	Product Size (bp)
Arg-1	ATGGAAGAGACCTTCAGCTAC	GCTGTCTTCCCAAGAGTTGGG	224
iNOS	GGAATCTTGGAGCGAGTTGT	CCTCTTGTCTTTGACCCAGTAG	99
CD206	TGCAAAGCTATAGGTGGAGAGC	ACGGGAGAACCATCACTCC	164
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC	159
gp130	GAAACAAGGTGGGCAAATCAG	CACCCAGAGCAGGTTATCTTT	116
CD115	TCATTCAGAGCCAGCTGCCCAT	ACAGGCTCCCAAGAGGTTGACT	560

STAT3	CAAGCCTTTCCTGACA GAGG	AGACAATGTCCTCACT GCCC	221
STAT6	CATCTGAACCGACCA GGAACT	CTCTGTGGGGCCTAAT TTCCA	135
IL-6R	TGGGACCCGAGTTACT ACTT	TGGATGACGCATTGGT ACTG	110
β-actin	AGAGGGAAATCGTGC GTGAC	CAATAGTGATGACCT GGCCGT	138

2.13 Western blotting analysis

Briefly, cells were lysed in RIPA lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150mmol/L NaCl, 10 mmol/L phenylmethylsulfonylfluoride, 1 mmol/L EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate) for 30 to 40 mins on ice. Protein concentrations were determined using PierceTM BCA protein assay kit (Thermo Scientific, Rockford, USA). Proteins were resolved by SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, Massachusetts, USA). Membranes were blocked for 1 hour at room temperature in 5% skim milk in 0.1% Tween 20 Tris buffered saline (TBS) and then incubated overnight with rabbit polyclonal antibodies to IL-6Rα (20μg/ml, Sino Biological Inc, China), phosphor-STAT6 (1μg/ml, R&D systems), STAT-3 (1:1000, Cell Signaling Technology, QLD, Australia), phospho-STAT3 (1:1000, Cell Signaling Technology), β-actin (1:1000, Abcam) and goat polyclonal antibodies to STAT6 (1μg/ml, R&D systems) and gp130 (1:500, R&D systems).

Membranes were then incubated with appropriate anti-goat or anti-rabbit IgG-HRP secondary antibodies (all at 1:2000, Santa Cruz) for 1 hour at room temperature. Membranes were washed again, exposed to Western Lightning Plus Enhanced Chemiluminescence Solution (PerkinElmer, Woodbridge, ON) for 1min, exposed to Amersham Imager 600 (GE Healthcare Life Sciences, New South Wales, Australia) for 5 seconds to 10 mins and developed using an

automatic film developer. The density of the specific bands was quantified using Image J software (National Institutes of Health, Bethesda, Maryland, USA).

2.14 Statistical analysis

Statistical evaluation of liver weight, limiting dilution, RT-PCR, and immunohistochemical analysis results were presented as Mean ± standard error of the mean (SEM), or Mean ± standard deviation (SD), as appropriate. Results were analysed using GraphPad prism 5 software (Graphpad Software, San Diego, USA) by unpaired Student's *t* tests and Mann-Whitney tests for samples with unknown and potentially disparate variances, or by one-way ANOVA followed by post-hoc analysis using Tukey's test with $p < 0.05$ accepted as a level of statistical significance.

A list of buffers prepared in the laboratory and indicated within this chapter is listed below.

10 x PBS

80 g NaCl (Sigma-Aldrich®, S5886)

14.4 g $Na_2HPO_4H_2O$ (Sigma-Aldrich®, S5136)

2.4 g KH_2PO_4 (Sigma-Aldrich®, P0662)

2 g KCl (Sigma-Aldrich®, P9541)

H_2O to 1L (pH 7.4 on dilution)

FACS buffer

100 ml 10 x PBS

1g BSA

2ml 10% Sodium Azide (Sigma-Aldrich®, S2002)

H_2O to 1L

HBSS

8 g NaCl (Sigma-Aldrich®, S5886)

14.4 g $Na_2HPO_4H_2O$ (Sigma-Aldrich®, S5136)

2.4 g KH_2PO_4 (Sigma-Aldrich®, P0662)

0.4 g KCl (Sigma-Aldrich®, P9541)

H_2O to 1L (pH 7.4 on dilution)

RBC Lysis Buffer

4.55 g NH_4Cl (Sigma-Aldrich®, S0171)

10ml 1 M HEPES (Sigma-Aldrich®, H3375)

H_2O to 500ml

SDS Running Buffer

25 mM Tris-base

192 mM Glycine

0.1% SDS

H_2O to 1L

Electroblotting transfer Buffer

25 mM Tris-base

192 mM Glycine

20% (v/v) ethanol

H_2O to 1L

Cell Lysis Buffer (RIPA)

50 mM Tris-HCl

150 mM NaCl

1% NP-40

1% Sodium Deoxycholate

0.1% SDS

Protease inhibitor tablets

H_2O to 10 ml

Tris Buffered Saline-Tween 20 (TBS-T)

150 mM NaCl

25 mM Tris-base

0.2% Tween-20

H_2O to 1L

Chapter 3.

Absence of TNF leads to liver infection during *L. major*-induced cutaneous leishmaniasis

3.1 Introduction

Cutaneous leishmaniasis, the most common form of leishmaniasis, infects a significant number of people worldwide. It causes skin lesions, which can persist for months or even years. The lesion evolves from papules into an ulcerative lesion but the infection remains non-fatal in most cases [1]. *L. major* is one of the most common old world species causing cutaneous leishmaniasis [152], and typical features of clinical manifestations as seen in patients are reflected in inbred mice of different genetic backgrounds [153]. The murine model of *L. major* infection is therefore, considered ideal for deciphering the role of different cytokines in response to infection, as well as understanding the effect of parasite-specific Th1/Th2 $CD4^+$ T cell subpopulation determined resistance and susceptibility [154].

The control of *L. major* infection in the host is mediated by the cellular immune response which requires macrophage activation. Resident macrophages exhibit a high level of phagocytosis to engulf the parasites, but the parasites take advantage of this process to internalize and replicate in the macrophage phagolysosomes [155]. Although resident macrophages cannot effectively eliminate the parasites, their increased secretion of cytokines/chemokines including TNF, IL-6 and monocyte chemoattractant protein-1 (MCP-1) contribute to recruiting blood monocytes, which are necessary to boost the defensive immune response [156]. Recruited monocytes are the major component of increased effector cells endowed with high plasticity and heterogeneity in the tissue [49] and their activation determines the outcomes of infection.

Tumor necrosis factor, a pleiotropic cytokine, was named for its anti-tumor effect but has since been identified as a key effector of a broad range of biological activities. TNF has a proven association with host resistance against cutaneous leishmaniasis. The skin lesion enlarged

following application of anti-TNF antibody in resistant mice, and mice controlled the parasites and lesions when TNF was restored [157]. Similarly, deprivation of TNF led to *L. major*-infected BALB/c mice which had recovered following prophylactic sub-lethal irradiation succumbing to leishmaniasis again [158]. Also, TNF-activated macrophages are able to kill *Leishmania in vitro* [158]. TNF is not directly toxic to these pathogens, rather it synergizes with IFN-γ to elicit a strong NO response which is necessary for macrophage killing of parasites [9]. Mice treated with anti-TNF antibody showed increased numbers of parasites and an enlarged lesion which correlated with reduced iNOS production [159]. There was evidence showing that impairment of M2 macrophage activation delayed cutaneous leishmaniasis, and cutaneous reconstitution of TNF in B6.TNF$^{-/-}$ mice retrieved IFN-γ secretion, enhanced *Leishmania* antigen presentation and prolonged host survival [160]. Recently, TNF has been demonstrated to restrict IL-4-induced Arg-1 expression in myeloid cells, in a way that triggered NO synthesized by iNOS to kill the parasites [134].

Intriguingly, in addition to the fact that TNF is important for NO production and parasite killing, we previously found this cytokine may determine the outcome of *Leishmania* infection through regulation of monocyte/macrophage differentiation [95]. How TNF regulates this process has not yet been addressed. Resistant B6.WT mice without the TNF gene challenged with *L. major* developed uncontrolled cutaneous leishmaniasis and generated a monocytic population exhibiting M2 macrophage-like phenotype. Although a strong Th1 response was still observed in these B6.TNF$^{-/-}$ mice, this novel macrophage population harbored a large number of parasites and expressed low levels of iNOS [95, 133, 134]. Most of these researchers focused on the infection of skin or draining lymph nodes and the role of TNF in visceralization is still unclear [132]. Therefore, whether visceral infection is the cause of death in the host needs to

be investigated, which may lead to identify the role of TNF-mediated innate immune responses in organ-specific protection during leishmaniasis.

The aim of this chapter was to examine the effect of loss of TNF on liver infection in *L. major*-induced leishmaniasis. Given the observation that loss of TNF was associated with abnormal appearance of M2 macrophages in the lymph nodes, I proposed that in a dysregulated immune response, where TNF is absent, monocyte/macrophage differentiation process in the liver would also be impaired.

3.2 Results

3.2.1 Visceral liver infection was observed in *L. major*-induced cutaneous leishmaniasis in B6.TNF$^{-/-}$ mice

Significant hind paw edema was observed in both B6.WT and B6.TNF$^{-/-}$ mice from day 21 onwards. Footpad swelling of B6.WT mice stabilized at a moderate level within the first 35 days post-infection (p.i.), and subsequently, they developed resistance to the parasites and had reduced footpad swelling. In contrast, B6.TNF$^{-/-}$ mice failed to restrain the parasites, with uncontrolled footpad swelling and elevated parasite burden (Fig 3.1B). B6.TNF$^{-/-}$ mice showed not only increased swelling and parasite burden with the footpad but parasites were also in visceral organs such as liver (Fig 3.1A). Although there was no significant difference in body weight between B6.WT and B6.TNF$^{-/-}$ mice (data not shown), the liver was visually enlarged with, and its weight increased significantly in B6.TNF$^{-/-}$ mice compared to B6.WT mice from day 35 p.i. (Fig 3.1A &3.2A). Parasites were detected in the liver of B6.TNF$^{-/-}$ mice from day 21 onwards and showed unrestrained replication to more than 300,000 parasites per gram of liver tissue at day 42 p.i., while the liver of infected B6.WT mice remained essentially parasite free (Fig 3.2B).

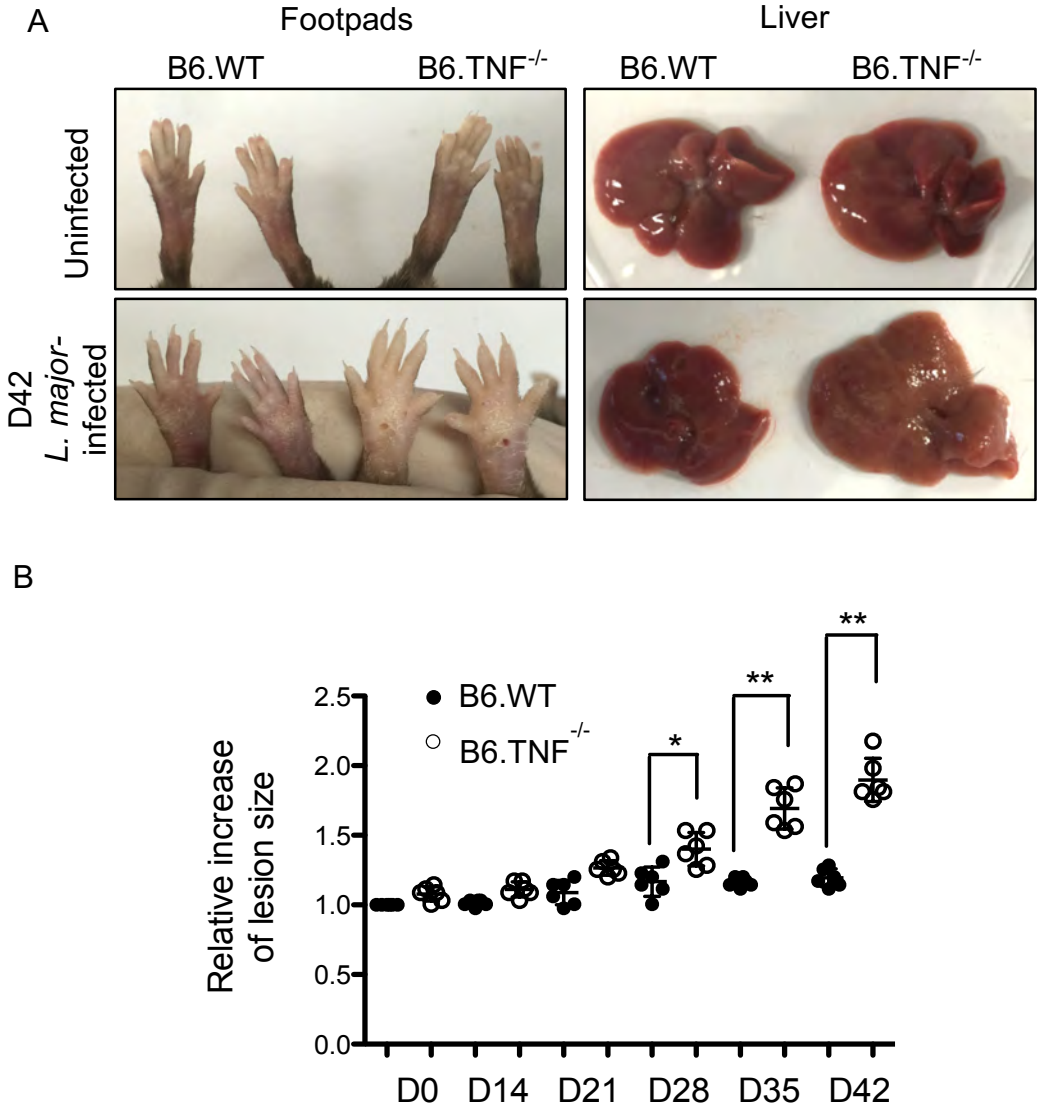


Figure 3.1 Liver enlargement and increased parasite burden in B6.TNF$^{-/-}$ mice

(A) Control and *L. major*-infected footpads and livers from B6.WT and B6.TNF$^{-/-}$ mice. (B) While the lesion and liver size and weight remained initially identical between B6.WT and B6.TNF$^{-/-}$ genotypes it increases significantly in footpads from day28 p.i.. Six B6.WT and B6.TNF$^{-/-}$ mice were used to determine the lesion size for each time point. Error bars represent the mean ± SD from one representative of three independent experiments. The *p* values were calculated using a two-tailed Mann-Whitney U test (*p<0.05, **p<0.01).

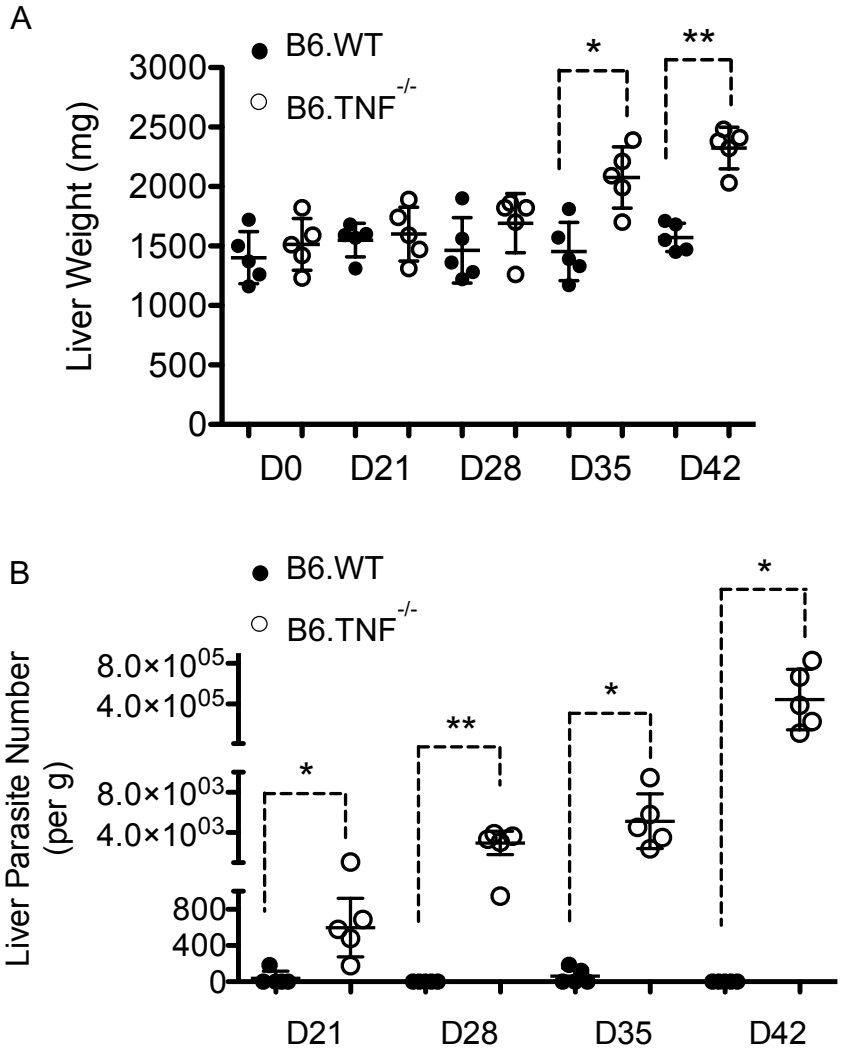


Figure 3.2 Liver enlargement and parasites were found in B6.TNF$^{-/-}$ mice.

(A) Control and *L. major*-infected livers from B6.WT and B6.TNF$^{-/-}$ mice. While livers were identical between B6.WT and B6.TNF$^{-/-}$ controls, B6.TNF$^{-/-}$ mice showed increased hepatomegaly from day 35 p.i. during *L. major* infection. Five B6.WT and B6.TNF$^{-/-}$ mice were used to determine the liver weight for each time point. (B) The number of viable parasites in the liver tissue of B6.WT and B6.TNF$^{-/-}$ mice were determined by limiting dilution analysis. The mean parasitic burden in the liver tissue of five mice is shown. One circle represents one animal (black: B6.WT; white: B6.TNF$^{-/-}$). The results were confirmed by the other two independent replications. All data are represented as mean ± SD. Significance was calculated using a two tailed Mann-Whitney U test. (*** p<0.001).

3.2.2 Discrete inflammatory foci in the liver of B6.TNF$^{-/-}$ mice during cutaneous leishmaniasis

In B6.WT and B6.TNF$^{-/-}$ mice, normal uninfected liver tissue consists of hexagonal hepatocytes radiating from the region of the central vein toward the periphery. From day 35 p.i., a point in time coinciding with a significant increase in liver weight and parasitic burden (Fig 3.2 A& B), abnormal liver structures such as swelling of hepatocytes and diffusely infiltrating inflammatory cells could be detected in *L. major*-infected B6.TNF$^{-/-}$ mice. Additionally, inflammatory foci appeared almost exclusively in infected gene-deficient mice (Fig 3.3). Weak hepatic expression of CD68 was observed in uninfected B6.WT and B6.TNF$^{-/-}$ groups, but day 42 p.i. it was upregulated strongly compared to its B6.WT counterpart. CD68^{+} cells were mainly found in the area around the border of the inflammatory foci, as well as on cells infiltrated in the centre of foci. Taken together, these results suggested that infiltration of inflammatory cells and hepatic inflammation are significantly elevated in B6.TNF$^{-/-}$ mice, compared to the corresponding B6.WT mice. Interestingly, inflammatory foci were observed in B6.TNF$^{-/-}$ mice but not in B6.WT mice (Fig 3.3 & 3.4A), and the number of inflammatory foci was significantly higher in B6.TNF$^{-/-}$ mice comparing to that in B6.WT mice (Fig 3.4B).

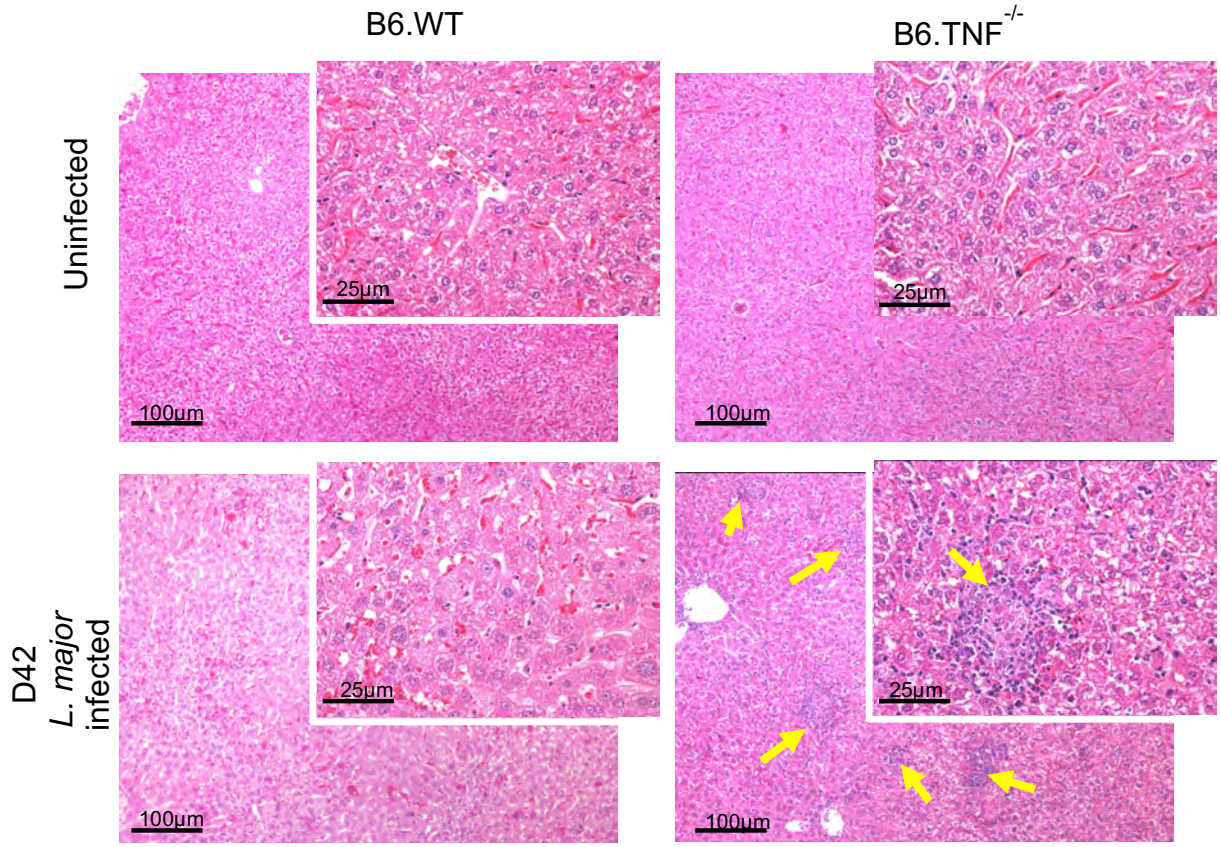


Figure 3.3 H&E staining on the liver tissue from B6.WT and B6.TNF$^{-/-}$ mice

Representative H&E stain liver sections of 15 B6.WT and B6.TNF$^{-/-}$ mice at low (10x) and high power (40x) are shown before infection and at day 42 after infection. There was no visible change of liver between B6.WT and B6.TNF$^{-/-}$ mice before infection (Upper panel). After day 42 p.i. (bottom panel), our analysis revealed a less organized liver structure in B6.TNF$^{-/-}$ mice with infiltration of inflammatory cells and the presence of inflammatory foci (arrowheads; Magnification 400 x).

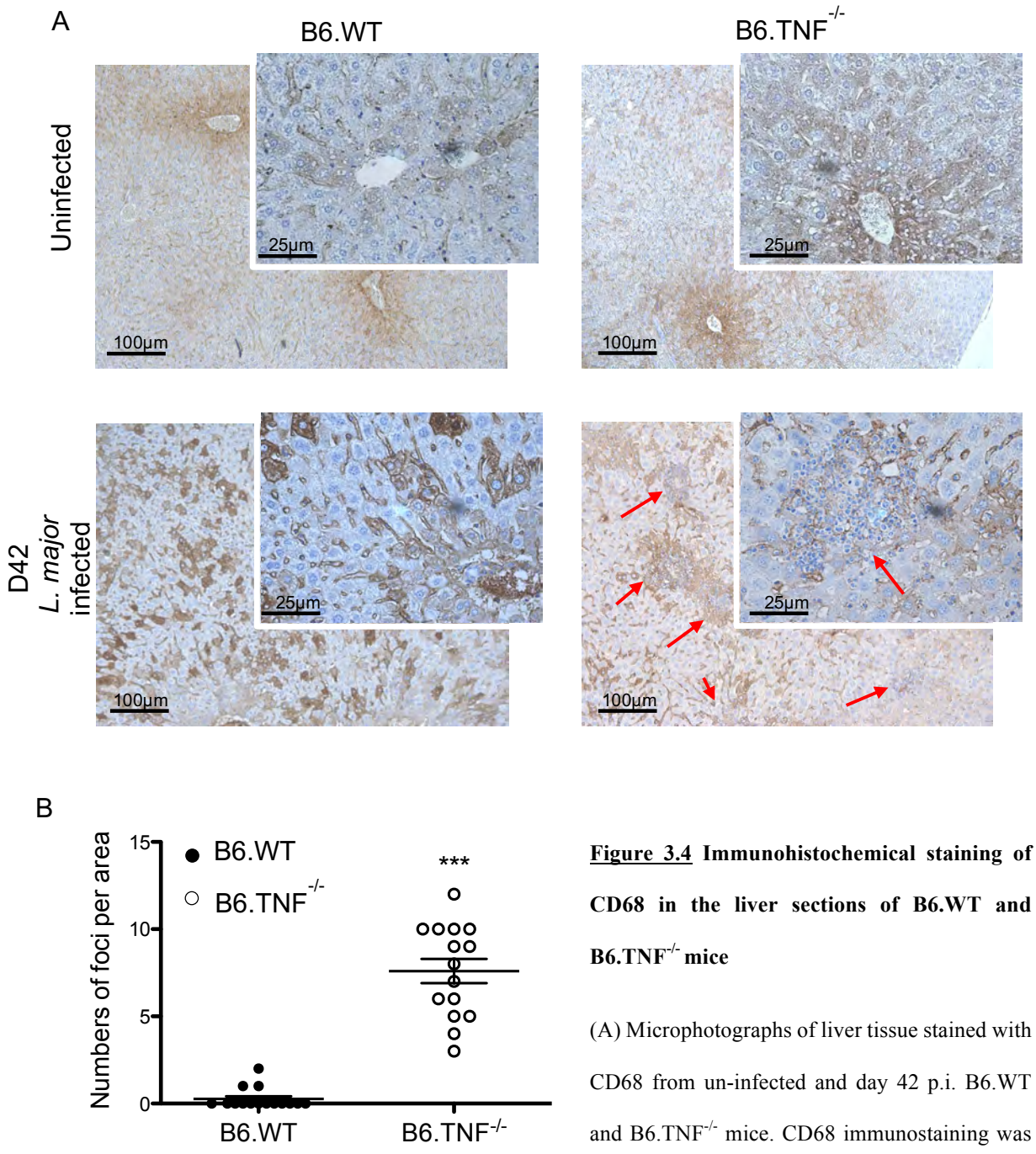


Figure 3.4 Immunohistochemical staining of CD68 in the liver sections of B6.WT and B6.TNF$^{-/-}$ mice

(A) Microphotographs of liver tissue stained with CD68 from un-infected and day 42 p.i. B6.WT and B6.TNF$^{-/-}$ mice. CD68 immunostaining was markedly increased in B6.TNF$^{-/-}$ at day 42 p.i. compare to that in B6.WT group. (B) Dispersion diagram showing the number of inflammatory foci per area, a very significant increase was observed in B6.TNF$^{-/-}$ group compared with levels in B6.WT group. One circle represents one animal, and the data are represented as mean ± SEM. The *p* values were calculated using two tailed Mann-Whitney U test (***p<0.001).

3.2.3 Cytokines levels in serum of B6.WT and B6.TNF$^{-/-}$ mice after infection

Cytokines levels in the serum can reflect the severity of disease progression [161, 162]. Therefore, we next evaluated the levels of different cytokines in serum from B6.WT and B6.TNF$^{-/-}$ mice. Quantitative detection of soluble cytokines by flow cytometry is a way to establish the immune response balance that closely reflects the immuno-regulation status *in vivo*. Here, we used a CBA flow cytokine array, to screen the serum cytokine levels of IL-6, IL-10, MCP-1, IFN-γ, TNF and IL-12p70 in *L. major* infected B6.WT and B6.TNF$^{-/-}$ mice, compared to uninfected mice.

Serum levels of MCP-1, IL-6 and IFN-γ were significantly increased in B6.TNF$^{-/-}$ mice compared to B6.WT mice (Fig 3.5). High levels of MCP-1 were detected in B6.TNF$^{-/-}$ mice after day 28 p.i. (Fig 3.5A) indicating that *L. major*-induced inflammation increased the potential to recruit monocytes. IL-6, a pro-inflammatory cytokine, was found to be increased by 17 fold at day 28 p.i. and 9 fold at day 35 and day 42 p.i. when compared to infected B6.WT mice (Fig 3.5B). Finally, a high concentration of IFN-γ (Fig 3.5C) is characteristic of a Th1-type immune response and the levels were determined to validate our data. Similar to previously published results [133], B6.TNF$^{-/-}$ mice showed a significant increase compared to that in B6.WT (day 28: mean ± SE, 269.45±163.56 versus mean ± SE, 3.50±1.49; day 35: mean ± SE, 668.39±424.14 versus mean ± SE, 3.50±2.46; day 42: mean ± SE, 766.03±622.3 versus mean ± SE, 6.14±4.11). Serum concentration of IL-10, TNF, and IL-12p70 remained below the detection limit for the assay in more than half of the serum samples throughout the course of infection (data not shown).

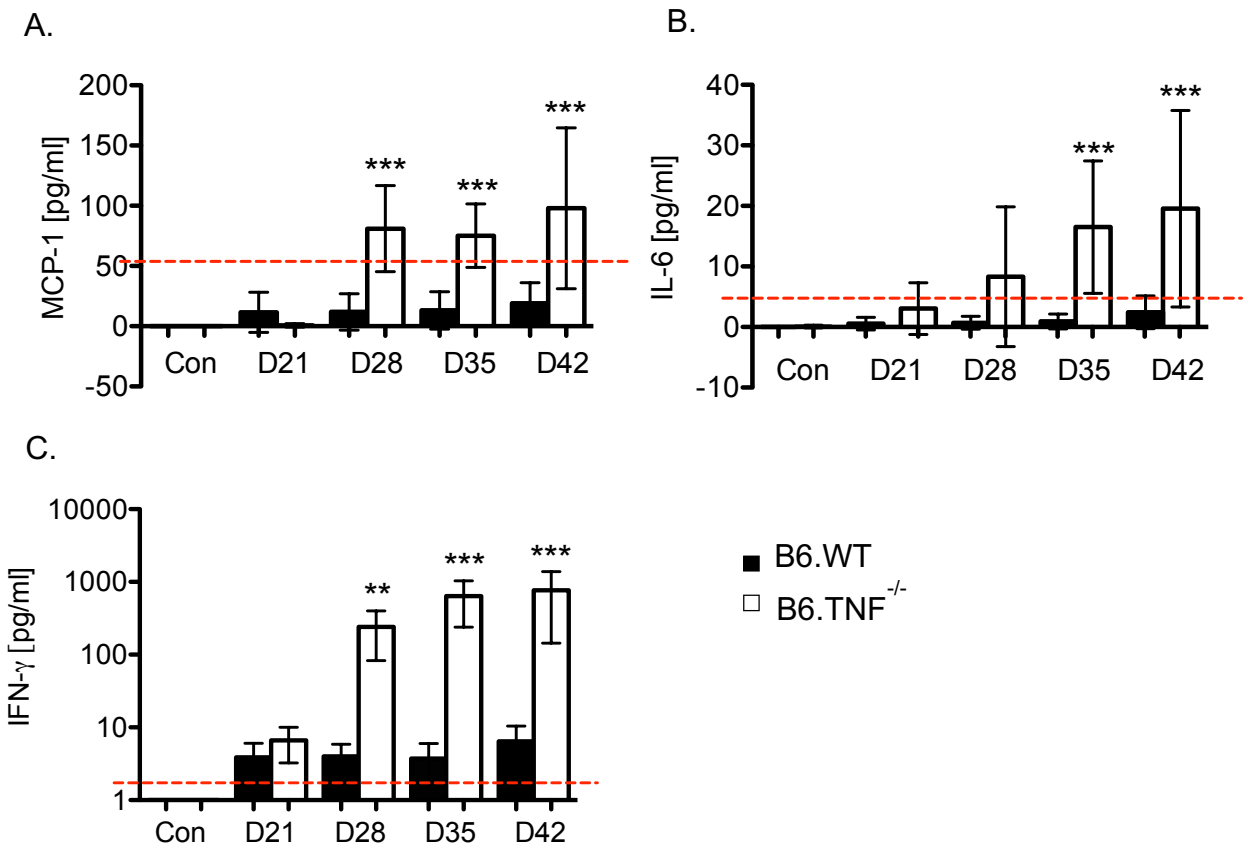


Figure 3.5 Cytokine secretion as measured by Cytometric Bead Array using flow cytometry

Serum levels of (A) MCP-1, (B) IL-6 and (C) IFN-γ were measured during the course of *L. major* infection in B6.WT and B6.TNF$^{-/-}$ mice. The red dashed lines represent the limit of detection for each cytokine (IL-6 5 pg/ml, MCP-1 52.7 pg/ml, and IFN-γ 2.5 pg/ml). Concentrations were expressed and compared with the basal level found in B6.WT and B6.TNF−/− mice without infection. Each value represents the mean of three independent experiments and each experiment was performed in five mice. Error bars denote SD. The p-values were calculated using two tailed Mann–Whitney U-test (**p < 0.01, ***p < 0.001).

3.2.4 An accumulating monocyte-derived macrophage population emerges in the liver of *L. major*-infected B6.TNF$^{-/-}$ mice

H&E staining showed a significant number of inflammatory cells infiltrating in the liver of B6.TNF$^{-/-}$mice. Monocytes, which are normally referred to as the dominant cell population, were recruited to the site of infection and differentiated into either monocyte-derived dendritic

cells or macrophages to protect the host against infection. Therefore, to identify the main content of this population and to understand how the absence of TNF contributed to changes in the cell compartment in the liver, we undertook two different comprehensive flow cytometric analyses throughout the course of infection.

Considering the heterogeneity of macrophages in the liver, three distinct subsets have been defined based on their $CD45^{+}F4/80^{+}$ expression with additional markers. Kupffer cells are defined as $CD45^{+}F4/80^{+}Ly6C^{-}CD11b^{-}$, inflammatory monocytes are $CD45^{+}F4/80^{+}Ly6C^{hi}$ $CD11b^{low}$and Mo-M are defined as $CD45^{+}F4/80^{+}Ly6C^{low}CD11b^{hi}$ (Fig 3.6) [163]. The number of Kupffer cells was not significantly different between the genotypes over the course of *L. major* infection except for day 21 p.i.. At this point in time, $CD45^{+}F4/80^{+}Ly6C^{-}CD11b^{-}$ population was around 2x higher in B6.WT mice compared to $B6.TNF^{-/-}$ mice (Fig 3.7A & 3.8A). The number of inflammatory monocytes increased in correlation with the footpad swelling irrespective of the genotype. At day 42 p.i. the infiltrating monocytes started to decrease (B6.WT) or reached a plateau ($B6.TNF^{-/-}$) (Fig 3.7A & 3.8B). At the same time, Mo-M were elevated significantly in $B6.TNF^{-/-}$ mice. This population was barely detectable in B6.WT mice (Fig 3.7A & 3.8C) most likely due to a lack of inflammatory stimuli. To analyze a specific role of TNF in the accumulation of Mo-M we employed TNF-competent mice of the BALB/c strain which are susceptible to *L. major* infection and display progressive visceralization. (Fig 3.7B).

Also, infection-induced inflammation can lead to a large increase in DCs at the infection site. These DCs are derived from recruited monocytes, and not only produce iNOS and TNF to eliminate invaders, but also can control the induction of protective T helper 1 responses against

infection. Therefore, we investigated if the absence of TNF affects the differentiation process of Mo-DCs during *L. major*-induced liver infection. DCs, once mature, are characterized by high expression of CD11c. Thus, we examined the expression of CD11c based on the $CD45^{+}CD11b^{hi}Ly6C^{hi}$ population to examine DCs maturation. Mice of both genotypes had a very distinct $CD11b^{hi}Ly6C^{hi}$ population, and there was no significant difference in their number (Fig 3.9A). However, this population in B6.$TNF^{-/-}$ mice displayed a comparatively lower expression of CD11b and Ly6C, and its expression of CD11c was significantly lower than that in B6.WT mice (Fig 3.9B), which indicated Mo-DCs in B6.$TNF^{-/-}$ mice were not as mature as that in B6.WT mice.

Altogether, in *L. major* resistant B6.WT mice, the absence of TNF removed their ability to resolve the *L. major* infection, and showed liver infection similar to susceptible BALB/c mice. Although monocytes were recruited continuously into the liver, the differentiation process from monocyte to DCs was affected due to loss of TNF, and Mo-DCs were not as mature as those in the liver of B6.WT mice. Of note, Mo-M was observed continuously accumulating only in the livers of B6.$TNF^{-/-}$mice.

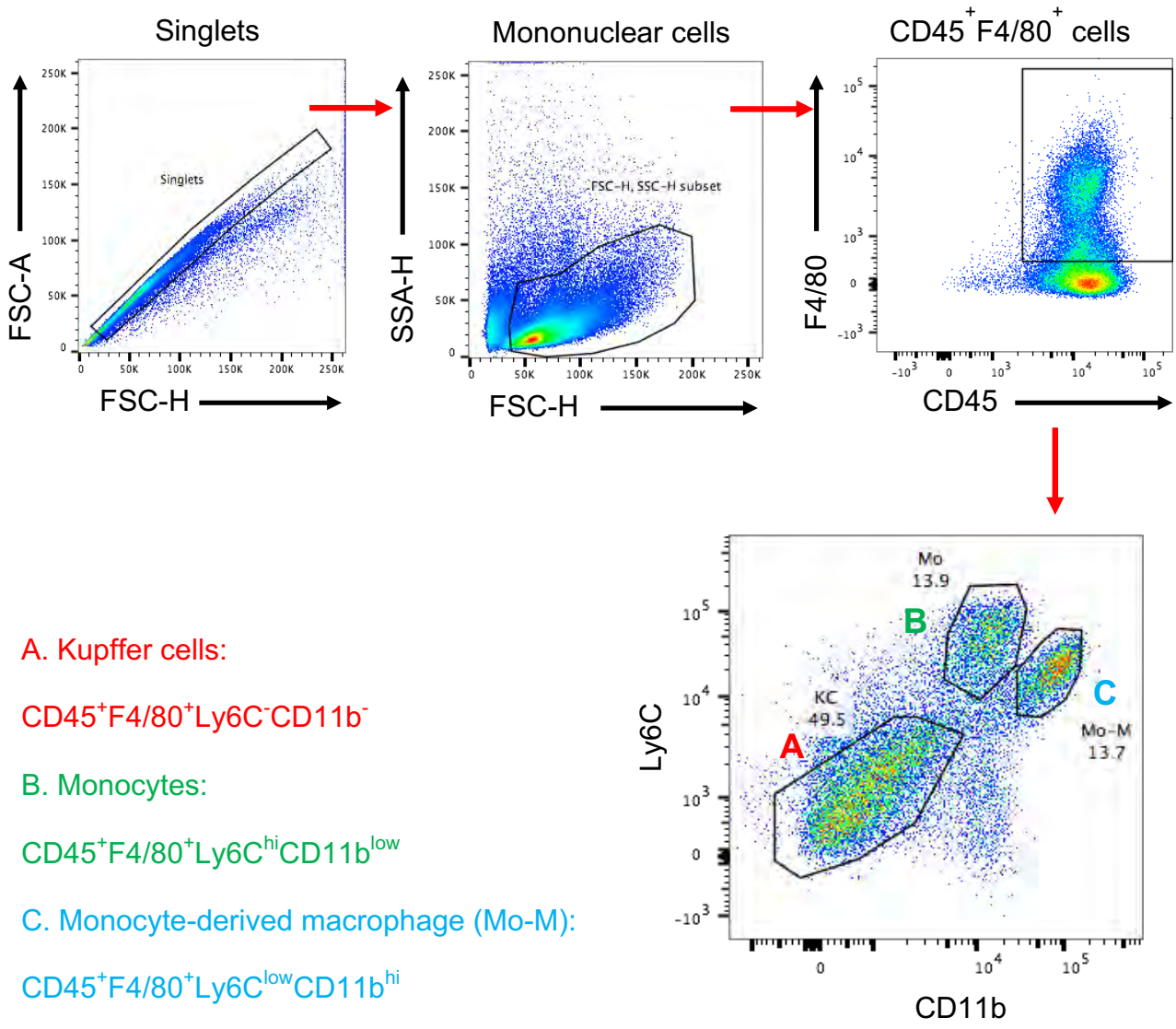


Figure 3.6 Gating strategy for different monocyte/macrophage population in the liver.

Representative images of three different monocyte/macrophage population in B6.TNF$^{-/-}$ mice is displayed. We defined population A as Kupffer cells, which are CD45^{+}F4/80^{+}Ly6C^{-}CD11b^{-}, population B as Monocytes, which are CD45^{+}F4/80^{+}Ly6C^{hi}CD11b^{low}, and population C as Mo-M, which are CD45^{+}F4/80^{+}Ly6C^{lo2}CD11b^{hi}.

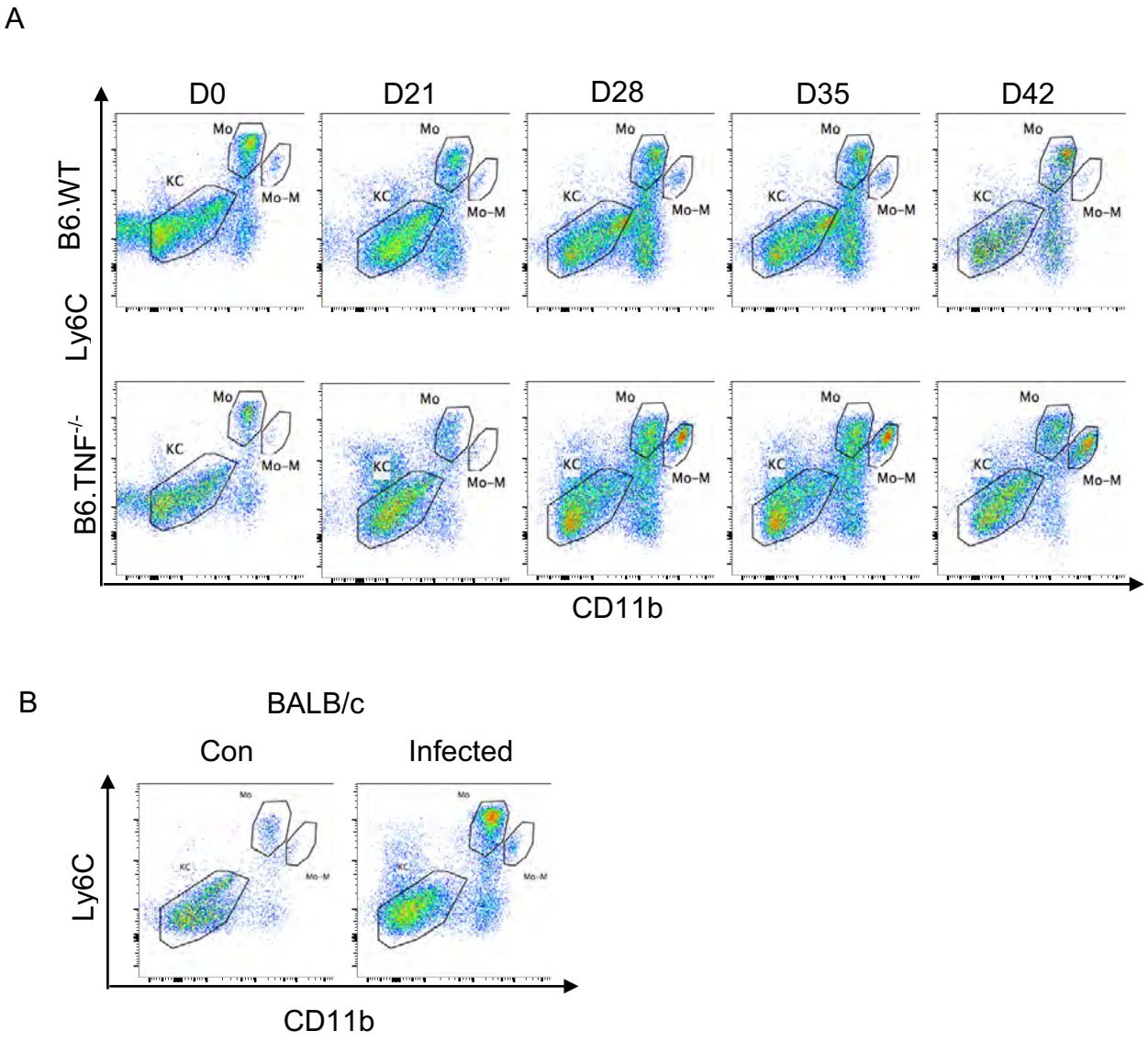


Figure 3.7 Mo-M population was only found in B6.TNF$^{-/-}$ mice.

Flow cytometry analysis revealed the changes of three different liver macrophage populations based on gated CD45^{+}F4/80^{+} cells from B6.WT and B6.TNF$^{-/-}$ mice in the course of *L. major* infection. Mo-M accumulated only in B6.TNF$^{-/-}$ mice in the course of *L. major* infection (A). Although BALB/c mice showed severe liver infection, Mo-M was rarely observed during leishmaniasis (B). A representative staining is shown, and results were confirmed by two independent experiments.

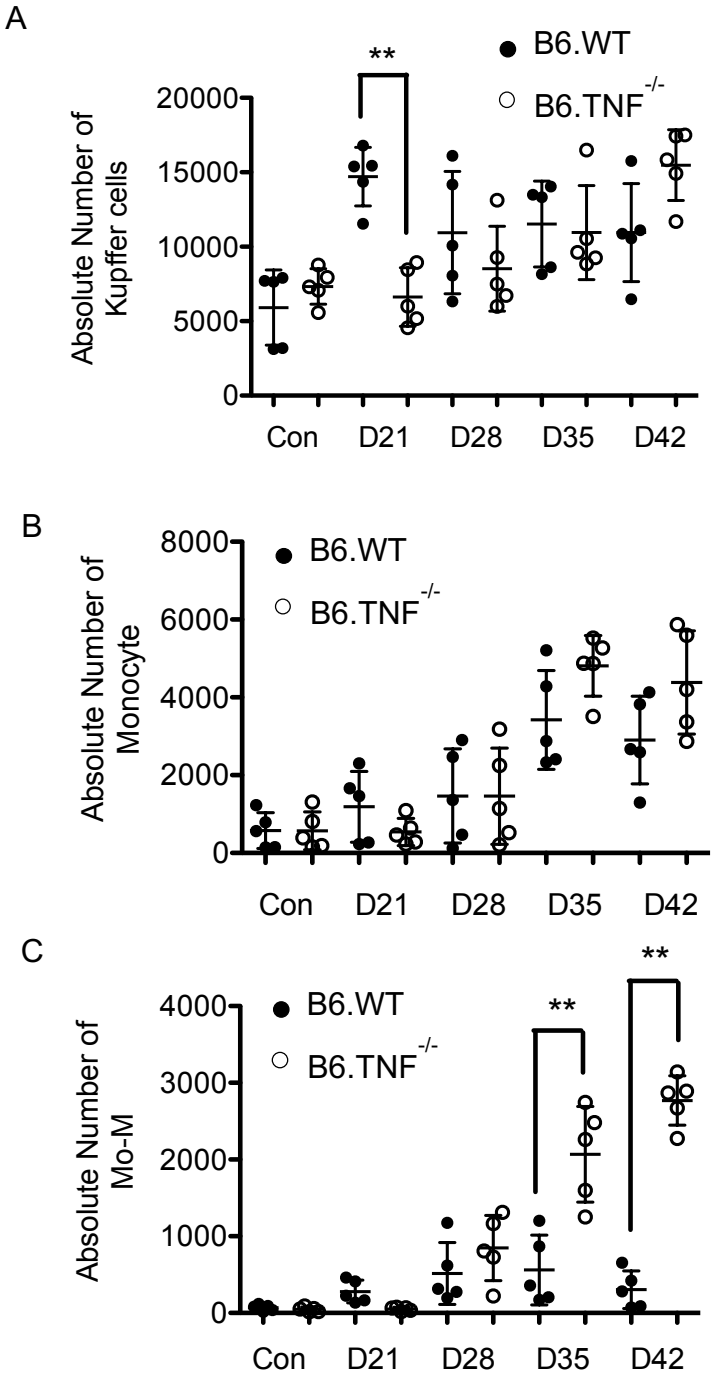


Figure 3.8 Quantification of three different cell population of B6.WT and B6.TNF$^{-/-}$ mice.

Quantification by flow cytometry of the total (A) Kupffer cells (B) Mo and (C) Mo-M from B6.WT and B6.TNF$^{-/-}$ mice in the course of *L. major* infection. Each error bar represents means ± SD from one experiment, and results were confirmed by two independent experiments. The *p* values were calculated using two tailed Mann-Whitney U test (**p<0.01).

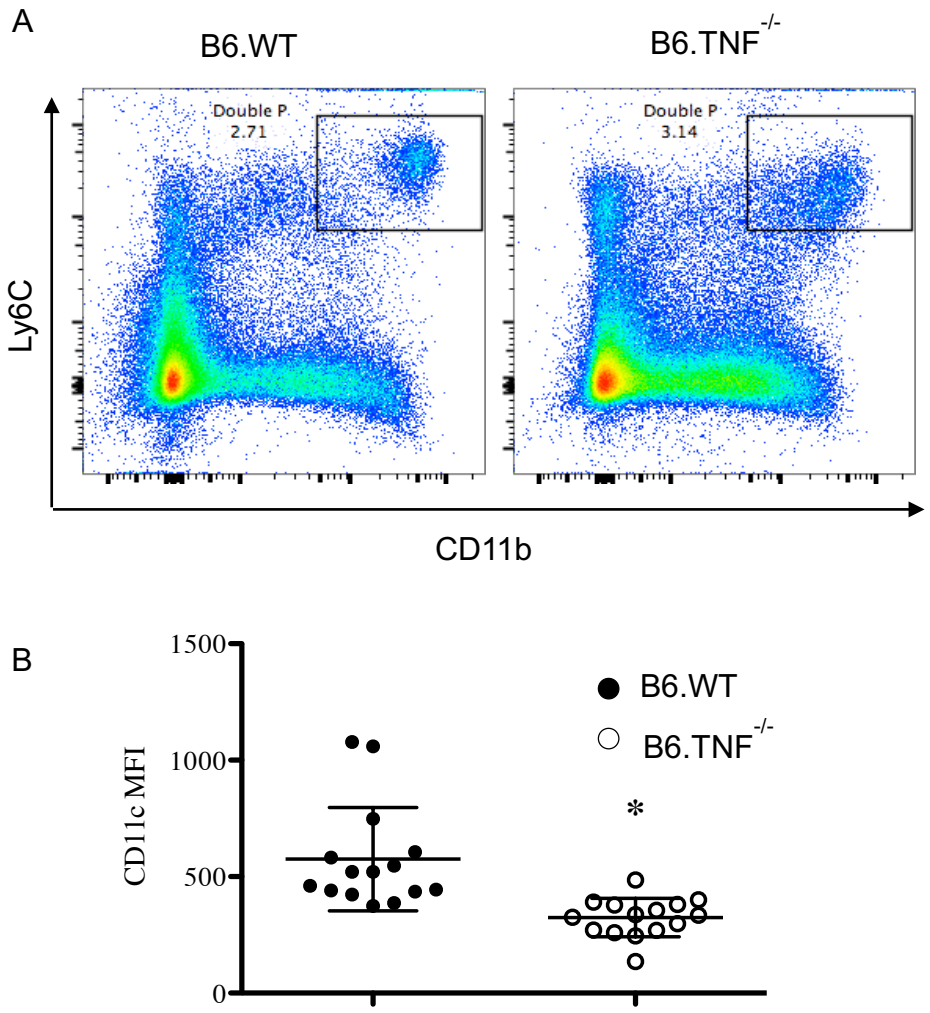


Figure 3.9 The expression of CD11c on monocytic cells from B6.WT and B6.TNF$^{-/-}$ mice

(A) Flow cytometry analysis of liver Ly6C^{+}CD11b^{+} cells obtained from *L. major*-infected B6.WT and B6.TNF$^{-/-}$ mice. The data (Means ± SEM) represented the results from five samples of three biological repeats. (B) Quantification by flow cytometry of the expression of CD11c on the Ly6C^{+}CD11b^{+} cells upon *L. major* infection. The data represent the mean intensity of fluorescence (MIF) of CD11c expression by Ly6C^{+}CD11b^{+} cells upon *L. major* infection. Results represent Means ± SEM, n=15. *p<0.05 comparing to B6.WT group, two tailed Mann-Whitney U test.

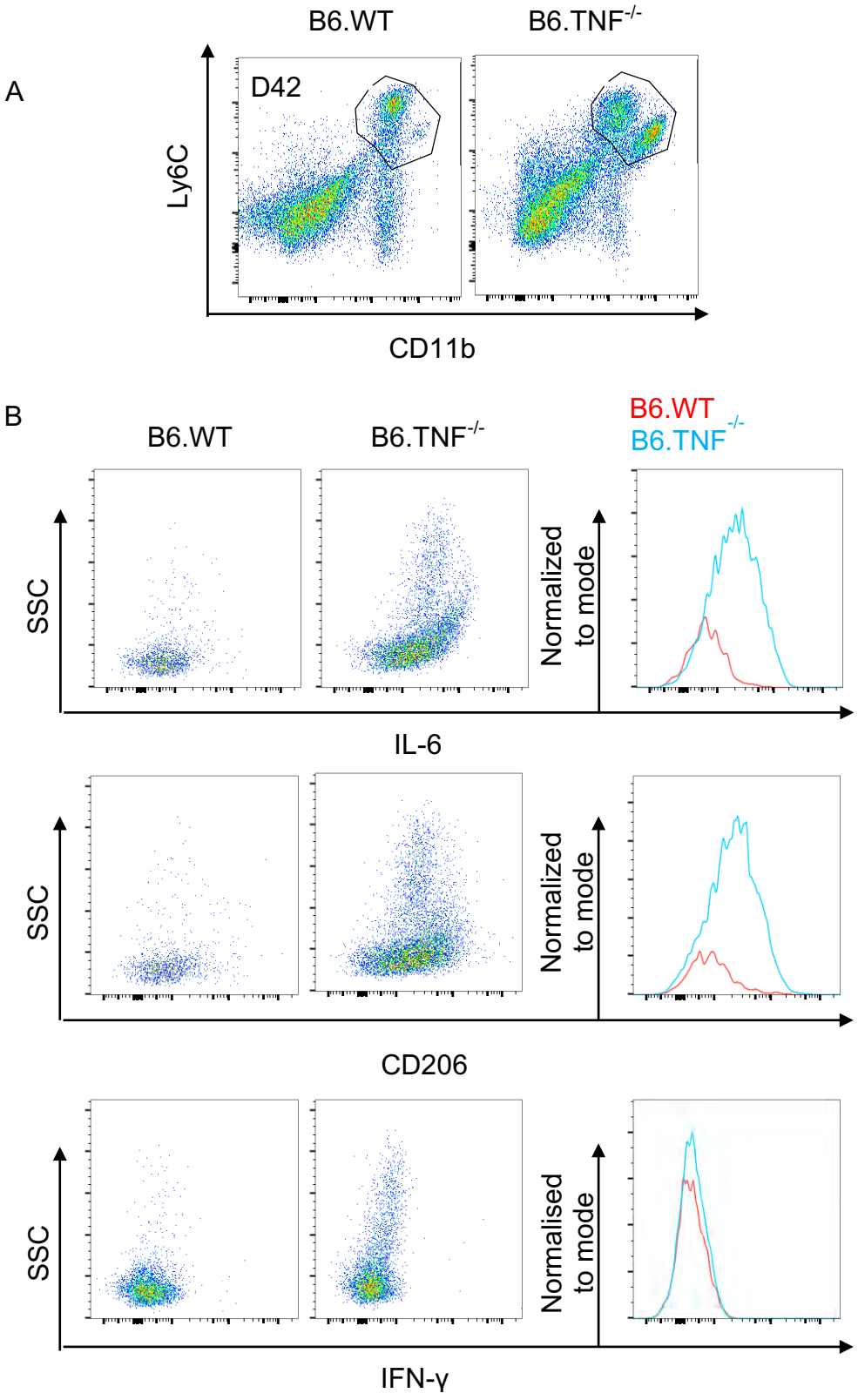
3.2.5 The Mo-M population in TNF$^{-/-}$ mice display an alternatively activated phenotype with high IL-6 expression

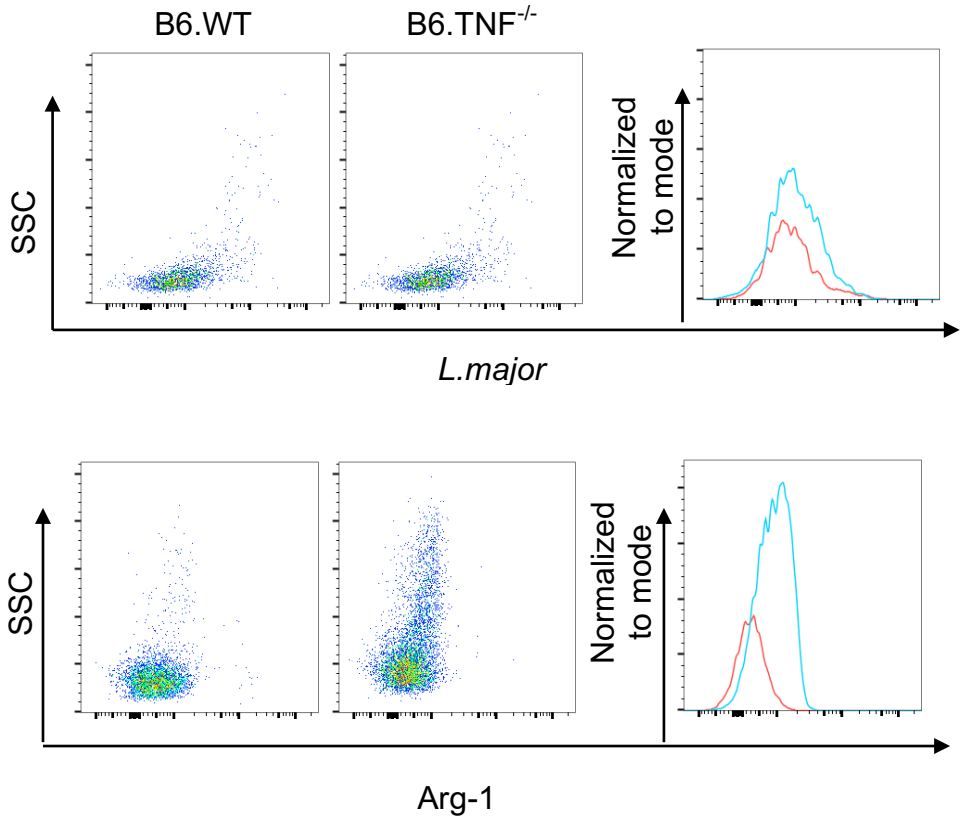
Macrophages differentiate into M1 and M2 phenotypes in response to microbial insults [164]. Both M1 and M2 can be induced in the course of leishmanial infection according to the microenvironment. M1 macrophages are activated by Th1 cytokines such as IFN-γ, with high-expression of leishmanicidal mediators such as NO, and effective control of *Leishmania* infection. By contrast, M2 macrophage differentiation is primed by Th2 cytokines, such as IL-4, and contributes to tissue repair but fails to eliminate parasites. As shown above (Fig 3.7A), B6.TNF$^{-/-}$ mice failed to clear the parasite from the liver and displayed an accumulation of a TNF-specific, unique Mo-M accumulation. To further investigate this Mo-M population in B6.TNF$^{-/-}$ mice during murine leishmaniasis, we followed the previously used gating strategy and combined both CD11b^{+}Ly6C^{+} populations since CD11b^{hi}Ly6C^{lo} are missing in B6.WT mice (Fig 3.10A). We characterized the phenotypes using intracellular flow cytometry of IL-6, CD206 and IFN-γ as control for the quality of the sort. The intracellular antigens were plotted against SSC (Fig 3.10B). Corresponding to the increased IL-6 secretion in serum of infected B6.TNF$^{-/-}$ mice, an increased level of IL-6 expression was detected in liver macrophages (Fig 3.10B). Although IL-6 is regarded as pro-inflammatory cytokine, it is also involved in the establishment of a Th2 response which in turn, could modulate the activation pathway of macrophage differentiation[165]. Additionally, the macrophage mannose receptor CD206 was strongly up-regulated (Fig 3.10B). There was no difference in the presence of IFN-γ between B6.WT and B6.TNF$^{-/-}$ mice because IFN-γ is normally produced by T cells rather than monocytes or Mo-M (Fig 3.10B). In summary, we found IL-6 and CD206 increased in the combined Mo and Mo- M of B6.TNF$^{-/-}$mice, indicating that this population may comprise large proportion of alternatively activated macrophages in B6.TNF$^{-/-}$ mice during *L. major* infection.

The detection of SSC^{hi} cells in B6.$TNF^{-/-}$ mice was striking and indicates a marked presence of cells with high granularity.

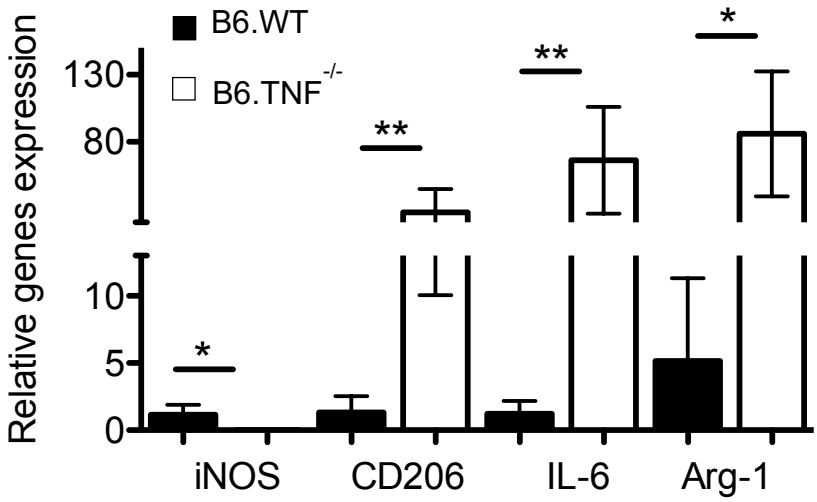
To further analyse Mo-M population in the liver during *L. major* infection, we isolated these cells and characterized their phenotype with regard to the gene expression of these marker molecules using qPCR. We confirmed our flow cytometry results and showed that IL-6 and the alternative activation markers including Arg-1 and CD206 were significantly more highly expressed in B6.$TNF^{-/-}$ mice compared to B6.WT mice. In contrast to previous results [95], in the liver iNOS expression was decreased significantly in B6.$TNF^{-/-}$ mice (Fig 3.10C).

Previously, it had been shown that $CD11b^{hi}$ $Ly6C^{lo}$ population harbored a markedly increased number of parasites in skin and draining lymph nodes [95]. Because infected cells with a large burden of parasites are fragile and difficult to detect using flow cytometry, we sorted the distinct monocyte and Mo-M populations from B6.WT and B6.$TNF^{-/-}$ mice and conducted a staining with Diff-Quik. There was no distinctive morphological difference between monocyte and Mo-M cells. In B6.WT mice there were no visible parasite association with macrophages. By contrast, in both $CD11b^{+}Ly6C^{hi}$ and $CD11b^{+}Ly6C^{lo}$ macrophage populations isolated from B6.$TNF^{-/-}$ mice parasites could be detected association with macrophages (Fig 3.10D).


A
B6.WT
B6.TNF-/-
D42
Ly6C
CD11b
B
B6.WT
B6.TNF-/-
B6.WT
B6.TNF-/-
SSC
Normalized to mode
IL-6
SSC
Normalized to mode
CD206
SSC
Normalised to mode
IFN-γ


B6.WT
B6.TNF-/-
SSC
Normalized to mode
L.major
SSC
Normalized to mode
Arg-1

C


B6.WT
B6.TNF-/-
Relative genes expression
130
80
10
5
0
*
**
**
*
iNOS
CD206
IL-6
Arg-1

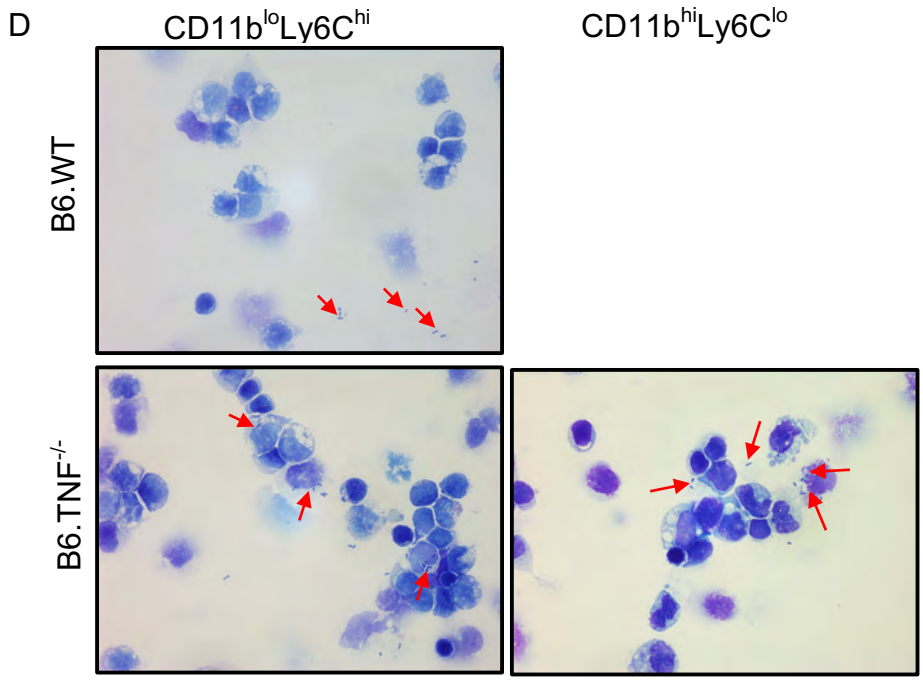


Figure 3.10 Phenotypic characterization of Mo-M in the liver of B6.TNF$^{-/-}$ mice.

(A) Gating strategy used in order to maintain a proper control for B6.TNF$^{-/-}$ mice. (B) The expression of IL-6, CD206, IFN-γ, *L. major* and Arg-1 were investigated in the combined population from B6.WT and B6.TNF$^{-/-}$ mice at day 42 p.i. (C) Gene expression of IL-6, CD206, iNOS and Arg-1 in the combined population of B6.WT and B6.TNF$^{-/-}$ mice at day 42 p.i. (n=5 per genotype, t-test, 3 times performed, Mean ± SD are shown). (D) Diff-Quik staining of monocyte and Mo-M isolated from B6.WT and B6.TNF$^{-/-}$ mice. Results represent one of three experiments. Red arrows represent *L. major* parasites.

3.2.6 The expression of iNOS and CD206 is correlated with parasite infection in the liver

In previous *in vitro* experiments, TNF has been demonstrated to be a central modulator of iNOS [134], but the regulation of its expression *in vivo* is more complex and less well defined. Therefore, we examined the localization of *L. major* parasite, $CD11b^+$ and iNOS in the liver of B6.WT and $B6.TNF^{-/-}$ mice. Mice from both genotypes showed a comparable $CD11b^+$ population during infection. A strong presence of *L. major* amastigotes could only be detected in $B6.TNF^{-/-}$ mice (Fig 3.11 & 3.12). B6.WT mice showed strong iNOS expression (Fig 3.11), while in the absence of TNF, iNOS expression was decreased similar to the situation in susceptible BALB/c mice (Fig 3.11). Upregulation of iNOS is normally linked to classically activated macrophage differentiation and antimicrobial properties, while an alternatively activated macrophage competitively utilizes L-arginine to generate Arg-1. Thus, owing to low levels of iNOS, we next sought to if theses $CD11b^+$ cells were M2 macrophage phenotype. In $B6.TNF^{-/-}$ mice, we observed stronger CD206 expression together with *L. major* in liver section (Fig 3.12). Thus, these results suggested more predominant M2 macrophages during leishmanial infection in the absence of TNF.

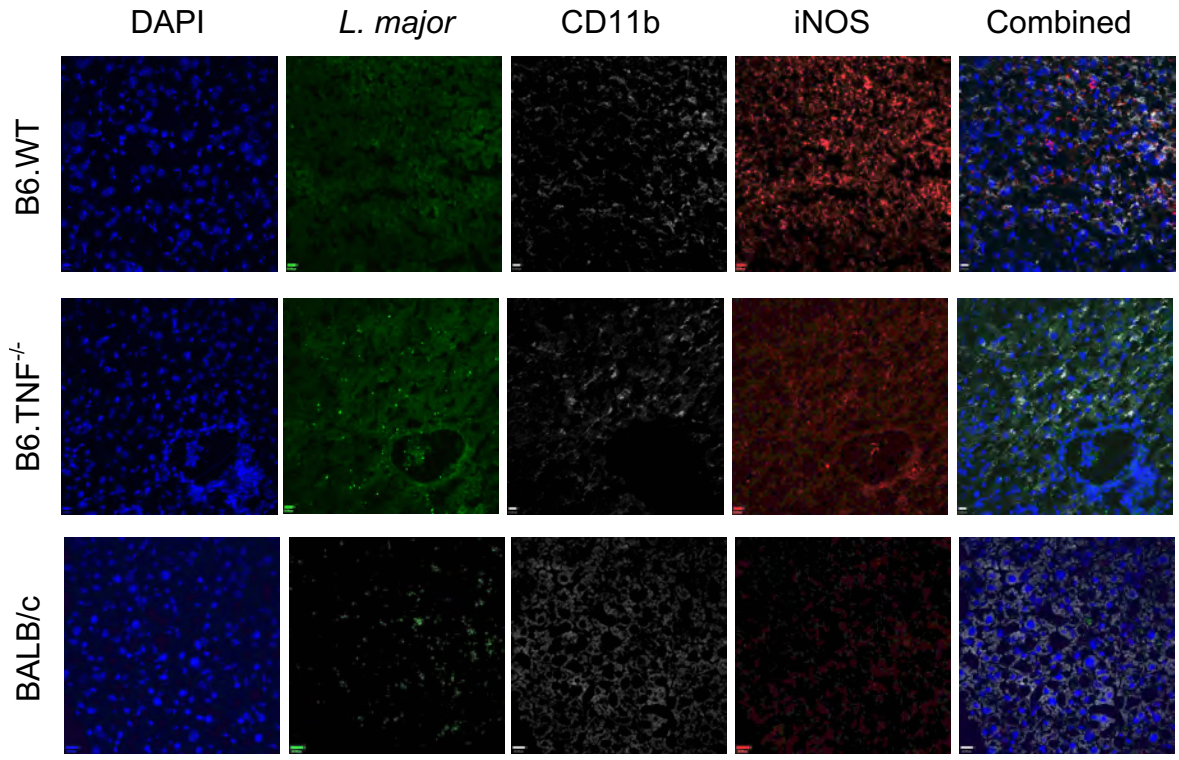


Figure 3.11 iNOS expression in B6.WT and B6.TNF$^{-/-}$ mice.

Overlay immunofluorescence staining of CD11b, iNOS and *L. major* in the liver tissue of B6.WT and B6.TNF$^{-/-}$ mice after day 42 p.i. BALB/c mice were used for positive control for *L. major* and iNOS staining. Green color for *L. major*, red color for anti-iNOS, grey for anti-CD11b and blue color from DAPI for visualization of nuclei. Results represent one of three experiments.

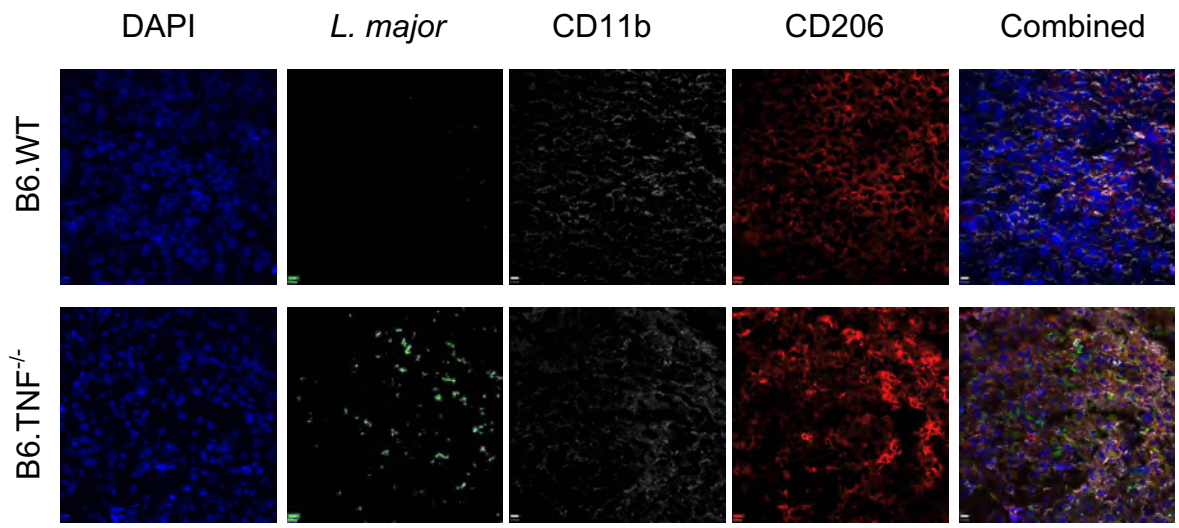


Figure 3.12 ***L. major*** **and CD206 expression in B6.TNF$^{-/-}$ mice.**

Immunofluorescence staining of CD11b, CD206 and *L. major* in the liver tissue of B6.WT and B6.TNF$^{-/-}$ mice after day 42 p.i.. Green color for *L. major*, red color for anti-CD206 or anti-iNOS, grey for anti-CD11b and blue color from DAPI for visualization of nuclei. Results represent one of three experiments.

3.3 Conclusion and discussion

The importance of TNF in controlling *Leishmania* infection has long been recognized. Loss of TNF in *L. major*-resistant mice results in susceptibility to leishmaniasis [95, 131-133, 158], and their IFN-γ-dependent NO production and innate immune cells recruitment, differentiation as well as activation also have been found impaired [9, 126, 134, 159, 160]. Interestingly, visceral organ infection such as liver infection was also observed in B6.TNF$^{-/-}$ mice during *L. major*-induced cutaneous leishmaniasis, but to date it has not been investigated. This chapter provides the first insights into liver infection, demonstrating that abnormal development of monocyte in the absence of TNF may be the reason for fatal visceral infection during *L. major*-induced cutaneous leishmaniasis.

Inoculation of *L. major* promastigotes into the hind paw of B6.WT mice resulted in swelling of footpads and cutaneous lesions, that typically peaked in size and number of parasites at day 28 p.i. and started to resolve spontaneously after day 35 p.i. [132]. However, B6.TNF$^{-/-}$ mice were found to be more susceptible to *L. major* infection [132]. As part of our investigation, we confirmed B6.TNF$^{-/-}$ mice failed to control parasite replication, and their footpads swelling persistently during the course of infection. Moreover, these mice developed severe liver infection. Livers from B6.TNF$^{-/-}$ mice were enlarged visually, and their weight increased significantly compared to B6.WT mice. Thus, these data adequately clarified that TNF is essential for restraining the parasite replication, controlling the disease progression, and inhibiting the development of severe visceralization during cutaneous leishmaniasis.

During visceral leishmaniasis, initial dominant reactive oxygen intermediates and iNOS response contribute to parasite resolution, but the most effective response is dependent on the

granuloma formation. It develops from infected Kupffer cells and then forms a mantle with recruited mononuclear cells to isolate and kill parasites cooperatively [22]. In the current study, we did not find any mature granulomas formation in *L. major*-infected liver of B6.TNF$^{-/-}$ mice, which is in line with earlier studies that B6.TNF$^{-/-}$ mice were not able to form granulomas during infection [166]. However, there were predominant inflammatory foci consisting of a large number of mononuclear cells infiltrated in the liver, that could be reflected by high levels of MCP-1 secretion after day 28 p.i. in B6.TNF$^{-/-}$mice. Lack of TNF delayed the early induction of chemokines and impaired recruitment of leukocytes, but that could be compensated by an enhanced Th1-like T cell response [166]. Our previous results showed a strong Th1 response also existed in B6.TNF$^{-/-}$ mice during *L. major* infection [133]. IFN-γ is mainly produced by T cells, and especially CD4^{+} T cell-producing IFN-γ is essential for controlling parasites and host survival. In the present study, IFN-γ was found highly expressed, which confirmed that there was no impaired of Th1 response in the absence of TNF.

Macrophages are the most important cell component of the innate immune response. They act as the final host cells for *Leishmania* parasites as well as the most effective effector cells for parasite killing. In the liver, hepatic macrophages consist of Kupffer cells and monocyte-derived macrophages involved in pathogen defense. Kupffer cells are derived from embryonic cells and self-maintain independently from hematopoietic input under steady state [51]. They serve as sentinels, sensing insults and initiating inflammation [167]. It was demonstrated that Kupffer cells underwent necroptosis in early infection with *Listeria*, triggering recruitment of monocytes and initiating immune response [163]. Here, we defined three different monocyte/macrophage populations according to their precursors and expression of typical markers. Kupffer cells were F4/80^{hi}CD11b^{lo}Ly6C^{lo}, and did not show any significant changes

between B6.WT and B6.TNF$^{-/-}$ mice during the course of infection until day 21 p.i., where the number of Kupffer cells were higher in B6.WT mice compared to B6.TNF$^{-/-}$ mice. Increased numbers of Kupffer cells were reported in administration of chronic liver disease, which exhibited morphological signs of cell activation and produced high levels of pro-inflammatory cytokines [168]. Although Kupffer cells most likely contributed to liver inflammation and necrosis, their accumulation in B6.WT mice during leishmaniasis may contribute to early parasite elimination and prevent parasite diffusion [169]. Additionally, the onset of immune response was proved to be impaired during cutaneous leishmaniasis in the absence of TNF [133], that may also explain the increased population appearing in B6.WT mice.

Monocytes, the circulating immune cell population, are normally recruited during inflammation. They infiltrate tissue and give rise to Mo-M and Mo-DCs in a process that depends on the specific tissue environment to determine the outcome. In the present study, the population of monocytes increased after day 35 p.i., which in line with the increased swelling of footpads in both group of mice. Monocytes began to decrease at day 42 p.i. in B6.WT mice, indicating the insult was resolved and no requirement of further recruitment of monocytes. However, in B6.TNF$^{-/-}$ mice monocytes were continuously recruited and subsequently generated into macrophages. This population only existed in the B6.TNF$^{-/-}$ strain. Although B6.TNF$^{-/-}$ mice recruited monocyte continuously, they failed to control and clear *L. major* parasites. This result is quite similar to that found in highly susceptible BALB/c mice, but monocyte-derived macrophages were not seen in infected BALB/c mice, which suggested accumulation of Mo-M was due to absence of TNF. Previously, a similar monocyte-derived macrophage population was observed in the draining lymph node of B6.TNF$^{-/-}$ mice during *L. major* infection. This was CD11b^{+}Ly-6C^{lo}CCR2^{lo}iNOS^{-} monocyte population exhibiting

alternative macrophage phenotype harbored large number of parasites [95]. Although we didn't find monocyte-derived macrophages containing a lot of parasites using flow cytometry in B6.TNF$^{-/-}$ mice, the parasites could be visualised by staining with Diff-Quik. In B6.WT mice there was no visible parasite association with macrophages. In contrast, in both CD11b^{+}Ly6C^{hi} and CD11b^{+}Ly6C^{lo} macrophage populations isolated from B6.TNF$^{-/-}$ mice had parasites which could be detected association with macrophages. Classically activated macrophages release pro-inflammatory cytokines that inhibit the proliferation of the parasites, while alternative activated macrophages have high phagocytosis capacity [170, 171]. Once engulfed by an alternative activated macrophage, *L. major* resident in phagocytic vacuoles thereby avoiding destruction [172]. Therefore, these results indicated monocyte differentiation was impaired due to absence of TNF. In B6.TNF$^{-/-}$ mice, monocytes preferentially differentiated into macrophages, which may present alternative activated macrophage phenotype, offering an explanation for increased number of Mo-M, and higher susceptibility and mortality of B6.TNF$^{-/-}$ mice.

Higher alternative macrophage marker expressed on monocyte and macrophages has been reported previously, recognized as a hallmark of leishmaniasis [93]. To determine the phenotype of this novel Mo-M, cell sorting and PCR was implemented. Isolated Mo-M clearly expressed high levels of M2 macrophage markers such as CD206 and Arg-1, but their iNOS expression decreased significantly, which were in accordance with the published literature. These data proved that Mo-M displayed an alternative activated macrophage phenotype and also indicated that the potential regulation ability of TNF in inhibiting alternative macrophage differentiation.

In addition, iNOS-dependent NO production is the main mechanism in macrophage of combating leishmaniasis [173]. iNOS shares the same substrate L-arginine with Arg-1, so competitive expression of iNOS and Arg-1 determines the regulation of macrophages. In the unique M2-like Mo-M, we found mRNA level of iNOS was competitively inhibited and Arg-1 was very highly expressed albeit we did not find these changes using flow cytometry. Besides, we also observed that in the liver tissue, iNOS expression was lower in $TNF^{-/-}$ mice within more *L. major* parasites dispersed, which indicated that absence of TNF led to the mice being unable to produce the leishmanicidal effector molecule NO and consequently to progressive visceralization of the parasites. This result differs from a study examining mouse draining lymph nodes. Phillips *et. al.* found production of iNOS was not affected, but an abnormal $CD11b^{+}$ cells strongly co-localized to areas with iNOS production and only presented in B6.$TNF^{-/-}$ mice [95]. This discrepancy is likely due to the difference in the method used to examine the target protein and strains variation between studies. In line with our findings are the published results which postulated a role of TNF in restricting Arg-1 expression which restored iNOS-mediated NO production during cutaneous leishmaniasis [134], which further supported our hypothesis that loss of TNF-induced aberrant monocyte differentiation lead to liver infection during *L. major* infection.

In addition, monocyte-derived DCs are also involved in the response to *Leishmania* infection. They produced a large amount of pro-inflammatory cytokines such as IL-12, generated iNOS and NO, and induced a protective Th1 immune response [94, 174]. In our present research, we did not find large numbers of $CD11c^{+}$ cells, which suggested monocyte-derived DCs is not the major population in this case. However, we still found higher CD11c expression in B6.WT mice based on the $F4/80^{lo}CD11b^{hi}$ $Ly6C^{hi}$ cells. The levels of CD11c expression reflects the

degree of DC maturation [175], and the less mature DCs found in the $TNF^{-/-}$ mice indicated absence of TNF may skew the differentiation from monocyte to macrophage/M2 macrophage, rather than DC, but further experiments are still required to resolve this.

Of note, a higher-level of IL-6 was observed in serum and Mo-M from B6.$TNF^{-/-}$ mice. IL-6 is usually a pro-inflammatory cytokine, but in certain circumstances can have an anti-inflammatory role. Increased levels of IL-6 as well as TNF were found in RA and SLE, and IL-6 blockade effectively reduced the disease progression [176]. Recently, it has been revealed that IL-6 can act as an anti-inflammatory cytokine to protect the tissue from an "overshooting" immune response [177]. Although IL-6 was reported is the key mediator in different diseases, the role of IL-6 in leishmaniasis is still controversial [178-181]. Current evidence suggested that IL-6, is elevated along with alternative activated macrophage in the absence of TNF, indicating IL-6 may play an anti-inflammatory role during leishmaniasis. Although further experiments are required to verify the anti-inflammatory role of IL-6 and determine how IL-6 affect the immune response during the course of infection, this is the first study revealing that IL-6 is negatively correlated with TNF in monocyte/macrophage differentiation during *L. major*-induced cutaneous leishmaniasis. This may indicate that TNF and IL-6 have a counterbalancing effect that may determine the development of leishmaniasis.

Overall, our results showed that loss of TNF induced hepatomegaly, which may be due to increased Mo-M. Although TNF inhibition of M2 macrophage development has been extensively reported in different circumstances [182-184], no convincing data directly showed TNF can act as an inhibitor of M2 macrophage during leishmaniasis. Absence of TNF impaired the normal monocyte differentiation process and generated a M2-like macrophage population,

which we propose can impede the protective immune response against *L. major*, although additional studies are still required to formally support our results.

Chapter 4.
The balance between TNF and IL-6 in regulation of monocyte/macrophage differentiation

4.1 Introduction

IL-6 is a pleiotropic cytokine which has a broad effect on a range of different cells and plays very important roles in regulation not only homeostasis but also inflammation. Initially, IL-6 was recognized as B-cell differentiation factor, which induced B cell maturation into immunoglobulin-secreting cells [136]. IL-6 can be produced by many different cells, and in turn can influence various types of cells through its unique IL-6 receptor (IL-6R) system. On the target cells, IL-6 binds to mIL-6R to form a complex which then associates with signaling transducing membrane glycoprotein (gp130) to transduce signaling [185]. This signaling through mIL-6R is named as classical IL-6 signaling. mIL-6R is expressed exclusively on the surface of hepatocytes, monocytes/ macrophage, neutrophils, epithelial cells and some leukocytes [186], thus classical IL-6 signaling only occur in these cells that express mIL-6R. Although most cells are not responsive to IL-6, they express gp130 and can bind to a complex of IL-6 bound to a soluble form of IL-6R, which is generated from mIL-6R via either proteolytic cleavage of their membrane moiety by alternative splicing [187], and this process is named as IL-6 trans-signaling which largely expands the effect of IL-6.

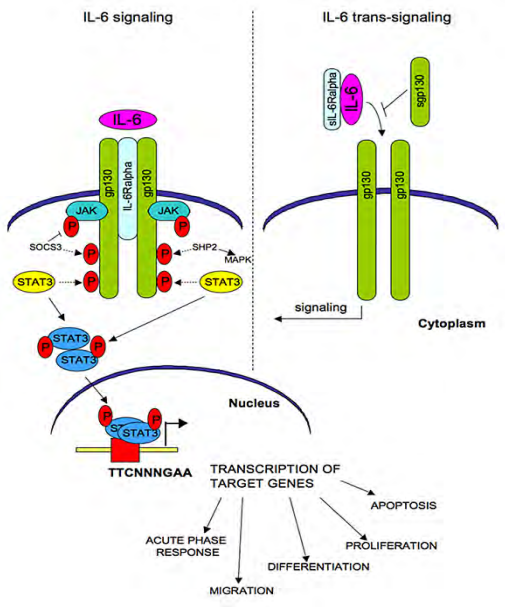


Figure 1.5 IL-6/IL-6R/gp130 signaling

IL-6 binds to mIL-6R trigger the homodimerization of gp130, and then a high affinity functional receptor complex of IL-6, IL-6R and gp130 is formed. The complex activates Janus kinases and JAK-mediated STAT3 phosphorylation in the nucleus, where it regulates transcription of target genes. Soluble IL-6R can also bind with IL-6 to form the complex with gp130 [188].

IL-6 is normally regarded as a pro-inflammatory cytokine, and its synergy with TNF reflects the severity of a series of autoimmune diseases [189, 190]. However, conflicting data recently reported that IL-6 displayed an anti-inflammatory feature, promoting M2 macrophage polarization and contributing to different diseases [146, 147, 177, 191]. Moreover, IL-6 was found to promote monocyte differentiation into macrophage rather than DCs in the presence of GM-CSF and IL-4 [192]. Although IL-6 is beneficial for some chronic inflammatory diseases, its role in leishmaniasis is not as straightforward as it appears in other diseases. IL-6 knockout mice were still able to generate the strong Th1 response and control the cutaneous infection as efficiently as their littermates, this indicated that IL-6 had little effect and is not required for the resolution of infection with *L. major* [179]. However, Wu *et al.* found the loss of IL-6 impaired lymphocytic response particularly $CD4^{+}$IFN-γ-producing cells when vaccination with CpG oligodeoxynucleotides and live *L. major* [181]. Compared to the inconsistent role of IL-6 during cutaneous leishmaniasis, its role in visceral leishmaniasis is quite clear and crucial. The expression of IL-6 was associated with the severity and complication of visceral leishmaniasis, and higher IL-6 was demonstrated to be negatively associated with TNF [193]. IL-6 was regarded as a hallmark of active visceral leishmaniasis in human, and the levels of circulating IL-6 were highly elevated in the patients' sera [194-197], and decreased after successful treatment [198]. Mice deficient of the IL-6 gene controlled the infection at the onset of progress. They assembled the granulomas and rapidly killed the parasite with development a strong Th1 response and IFN-γ production [199]. Therefore, it is difficult to illustrate the role and function of IL-6 due to different infection model and species of parasites in leishmaniasis.

As described in the previous chapter, IL-6 was elevated in mouse sera and monocyte-derived macrophage, with up-regulation of alternative activated macrophage markers, in *L. major*-infected B6.TNF$^{-/-}$ mice but not B6.WT mice. As a well-established DC maturation factor, TNF was found to have the potent ability to interfere with the action of IL-6 on the human monocytes [200]. TNF skewed the differentiation of monocytes from DC to macrophage by overriding the IL-6/M-CSF pathway [201], and a negative regulatory effect of TNF and IL-6 was reported against infection [202]. In light of results that IL-6 affected monocyte/macrophage differentiation in different diseases, it is hypothesized that if IL-6 is the inducer and promoter of alternatively activated macrophage in the absence of TNF, then the regulatory balance between IL-6 and TNF determines the disease outcome by modulation of the monocytes/macrophage differentiation process.

An *in vitro* cell culture system was used that allowed the investigation of the effect of cytokine in a controlled environment. In this chapter, the efficacy of IL-6 involved in monocyte, DC and macrophage differentiation was evaluated with or without TNF *in vitro*. Because of IL-6 elevation was found in B6.TNF$^{-/-}$ mice, cells treated with TNF inhibitor or cell isolated from B6.TNF$^{-/-}$ were also conducted here as positive controls. The most important purpose of these *in vitro* studies was to prove that monocyte/macrophage differentiation can be affected by the regulation of IL-6 and TNF.

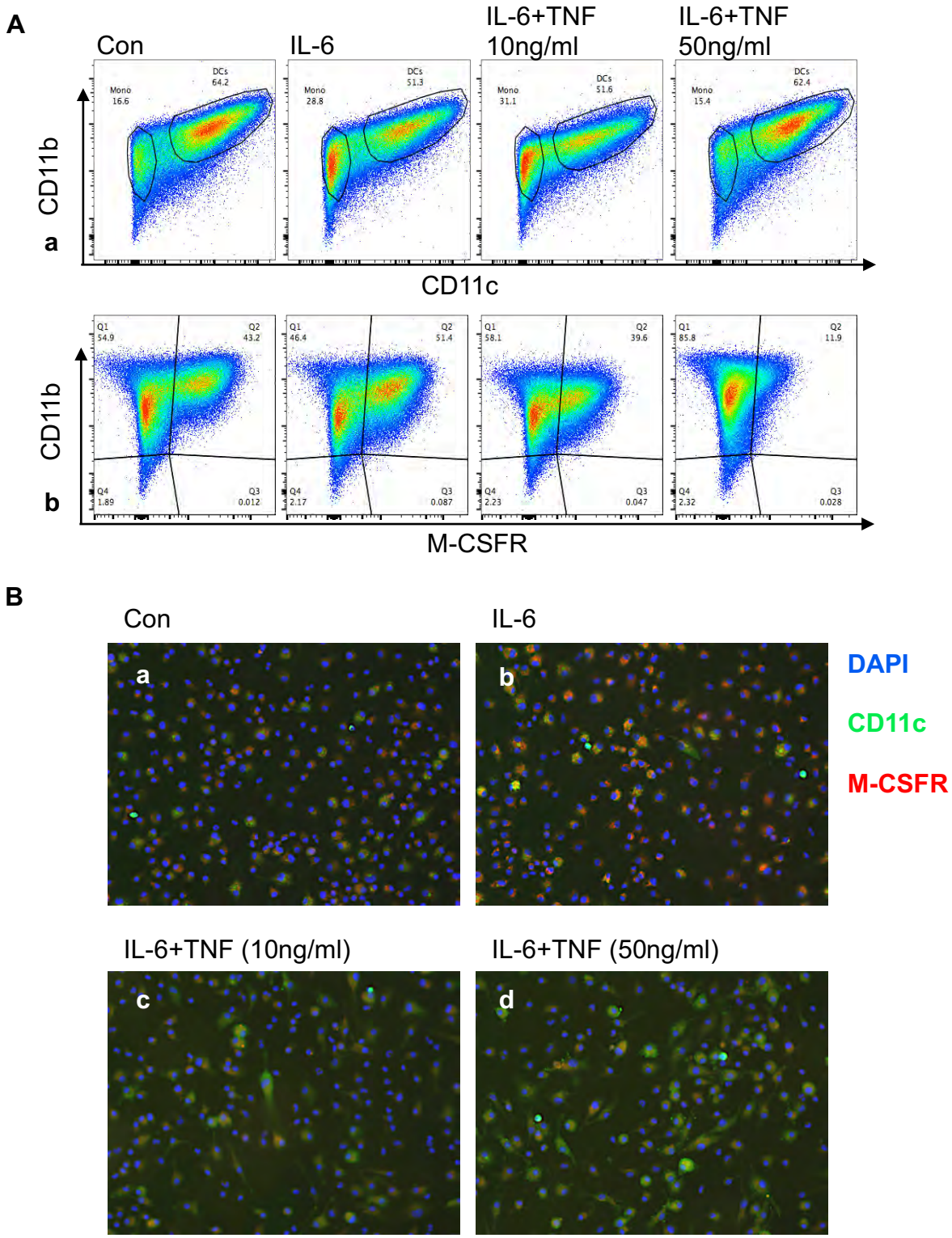
4.2 Results

4.2.1 TNF and IL-6 modulate the process of monocytes differentiation into macrophage or DC *in vitro*

Fibroblast cells co-cultured with human peripheral blood mononuclear cells can produce IL-6, and have been shown to switch differentiation to macrophage rather than DC, while TNF can reverse the process [201]. To investigate whether TNF and IL-6 affect mouse bone marrow-derived monocyte/macrophage differentiation, bone marrow cells from B6.WT mice were cultured with IL-4 and cell supernatant containing GM-CSF generating more than 60% $CD11b^{+}CD11c^{+}$DC. Adding IL-6 at day 4 of the bone marrow cell culture resulted in fewer cells with DC phenotype, and analysis of 3 independent experiments revealed that CD11c expression by these DCs was significant decreased compared to the control group. By contrast, the cells in the presence of IL-6 and TNF together restored the expression of CD11c indicating TNF reversed the differentiation process from monocytes to DC which was inhibited by IL-6 (Fig 4.1A-a).

We next examined the level of M-CSFR when treated with IL-6 and TNF. Adding IL-6 resulted in increased levels of M-CSFR compared to the control group which consistent with increased DC accumulation we found above. On additional exposure to TNF, IL-6-treated DC showed largely reduced M-CSFR level (Fig 4.1A-b). Similar results were found using immunofluorescence and qRT-PCR (Fig 4.1B & C). DC showed increased expression of M-CSFR when treated with IL-6 alone (Fig 4.1B-b), but CD11c expression was less than the control group (Fig 4.1B-a & -b). When DC were exposed to IL-6 and TNF together, the expression of M-CSFR was largely reduced, but CD11c was observed to be elevated, also

indicated by typical DC-shape cells (Fig 4.1B-c & -d). As activated monocytes spontaneously produce M-CSF, TNF might reverse the monocyte differentiation process via increased M-CSFR internalization to reduce IL-6-mediated macrophage generation.



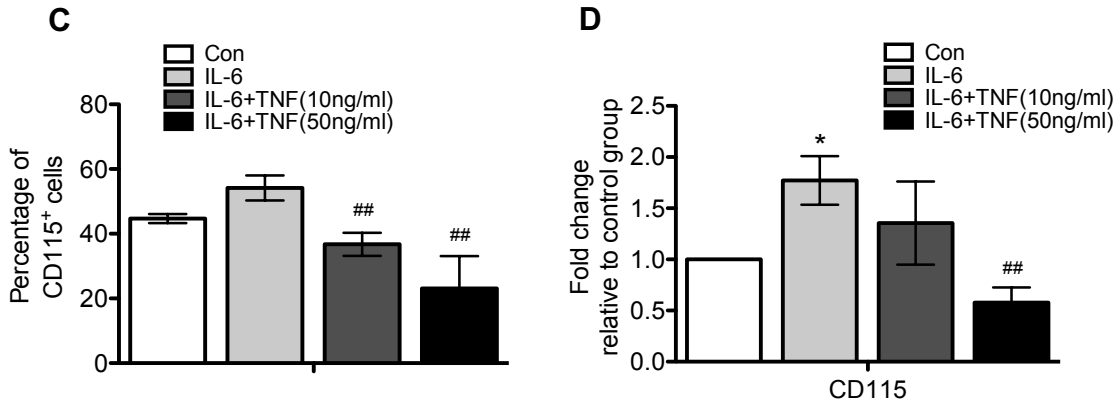


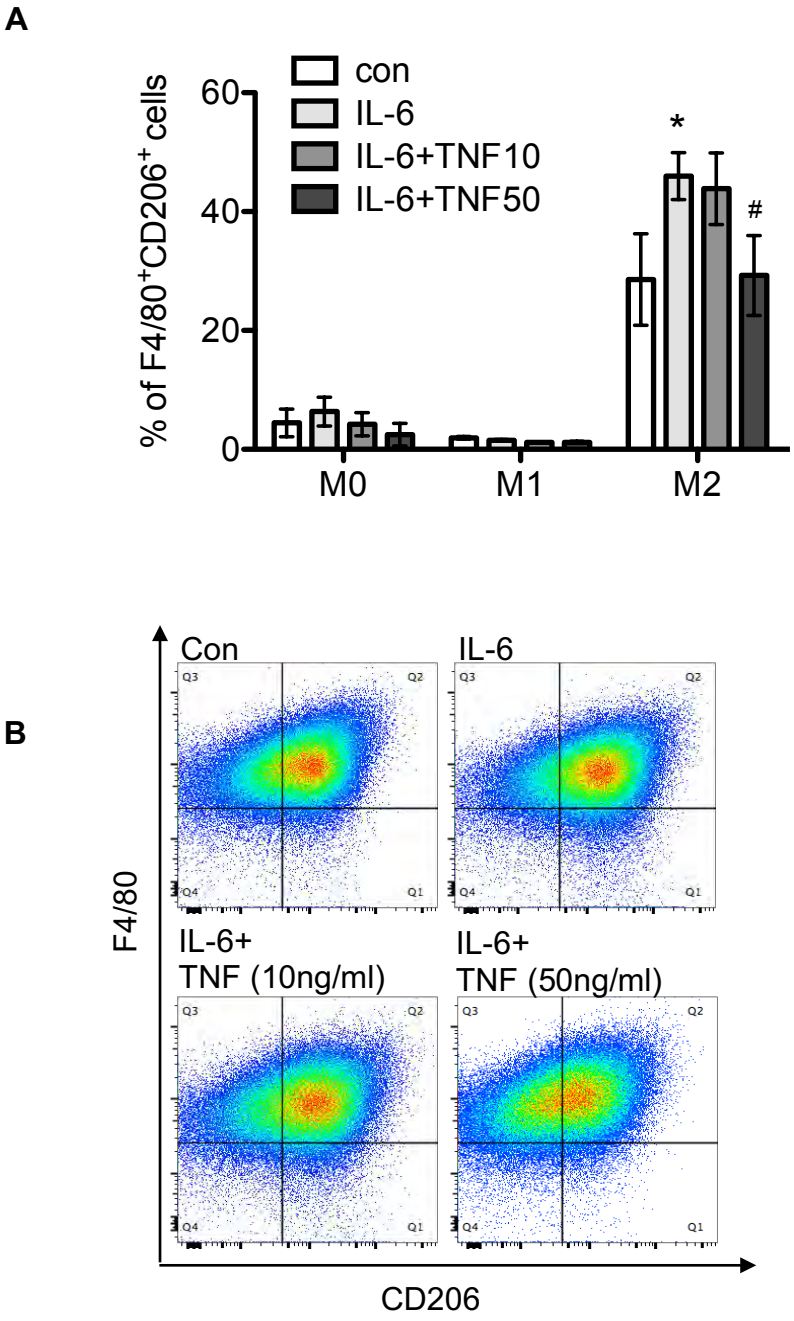
Figure 4.1 The effect of TNF and IL-6 in monocyte differentiation

(A) Bone marrow monocytes were cultured for 4 days in GM-CSF and IL-4. IL-6 and/or TNF was added from day 4. At day 7, differentiation was monitored using the expression of CD11b, CD11c and M-CSFR. (B) Immunofluorescence photographs represent monolayers of cells from each culture condition labelled for M-CSFR (red) CD11c (green) and nuclei (blue). (C) Quantification the results of a CD11b versus M-CSFR staining of three independent differentiation experiments. (D) qPCR data quantified M-CSFR gene expression in the cells treated with IL-6 and TNF. Results are representative of three independent experiments. Means ± SD were calibrated to median values of three experiments. The *p* values were calculated using one-way ANOVA and Tukey's post test. Statistically significant differences *$p<0.05$ comparing to control group, and ##$p<0.01$ comparing to IL-6-treated group.

4.2.2 A regulatory balance of TNF and IL-6 mediate M2 macrophage polarization

As TNF skewed IL-6-facilitated monocytes differentiation from macrophages to DCs, we next examined whether TNF and IL-6 affect the process of macrophage activation. Bone marrow derived macrophage were cultivated in the presence of M-CSF and harvested after 8 days, and are termed as M0 macrophages. M0 macrophages were further exposed to LPS and IFN-γ or IL-4 for 48 hours to generate M1 and M2 macrophages. Treatment of three different types of macrophages with IL-6 alone did not change proportions of M0 and M1 phenotype to be more "M2 like", based on the expression of the $F4/80^{+}CD206^{+}$ (Fig 4.2A and B). However, a significant increase of $F4/80^{+}CD206^{+}$ population was observed in M2 macrophage (Fig 4.2A & B). An analysis using qRT-PCR also revealed increased expression of CD206 and Arg-1 mRNA, and decreased iNOS mRNA expression (Fig 4.2C), which indicated IL-6 only enhanced IL-4-induced M2 macrophages differentiation. Furthermore, when applied as co-treatment with IL-6 and TNF, M0 and M1 proportions were still not affected based on the parameters assessed, compared to IL-6-treated group. The number of $F4/80^{+}CD206^{+}$ cells in the M2 macrophages was significantly reduced with increasing concentration of TNF, as well as the expression of CD206 and Arg-1 mRNA, but the iNOS mRNA expression was upregulated in the presence of TNF and IL-6 (Fig 4.2C). To further address if there is a regulatory effect between TNF and IL-6 in M2 macrophage activation, we used etanercept (Enbrel), which is a TNF inhibitor that neutralises murine TNF. After exposure to IL-4 for 48h, macrophage displayed the canonical features of M2 macrophage (Fig 4.3b). CD206 expression was upregulated, but the level of IL-6 did not show an obvious change in M2 macrophages from B6.WT mice. We found both CD206 and IL-6 increased in M2 macrophages from $B6.TNF^{-/-}$ mice (Fig. 4.3h), and their expression was elevated as compared to B6.WT macrophages. While macrophage pre-treated with increased concentration of etanercept and

then exposed to IL-4 not only upregulated the expression of CD206 but also increased the level of IL-6 (Fig 4.3c-f).



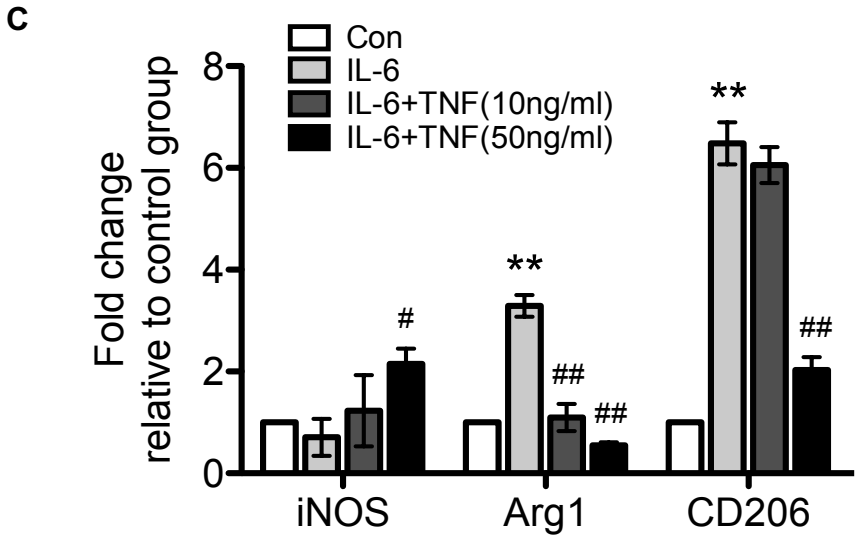


Figure 4.2 The effect of TNF and IL-6 in macrophage differentiation

(A) Bone marrow monocytes were cultured for 7 days and further exposed to different cytokines to generate separately M0, M1 and M2 macrophage cells. M0, M1 and M2 macrophage were incubated with IL-6 or IL-6/TNF, and differentiation was analysed based on the cells expressing F4/80 and CD206. All the data present as Means ± SEM calibrated to control group values of three experiments. The *p* values were calculated using one-way ANOVA and Tukey's post comparison test. Statistically significant differences $*p<0.05$ comparing to control group, and $^{\#}p<0.05$ comparing to IL-6-treated group. (B) Flow cytometry plots represent M2 macrophage from each culture condition labelled for F4/80 and CD206. (C) qPCR data revealed gene expression of iNOS, Arg-1 and CD206 in M2 macrophage treated with IL-6 and TNF. Results are representative of three independent experiments and represent as Means ± SD calibrated to control group. The *p* values were calculated using one-way ANOVA and Tukey's post comparison test., $**p<0.01$ comparing to control group, and $^{\#}p<0.05$, $^{\#\#}p<0.01$ comparing to IL-6-treated group.

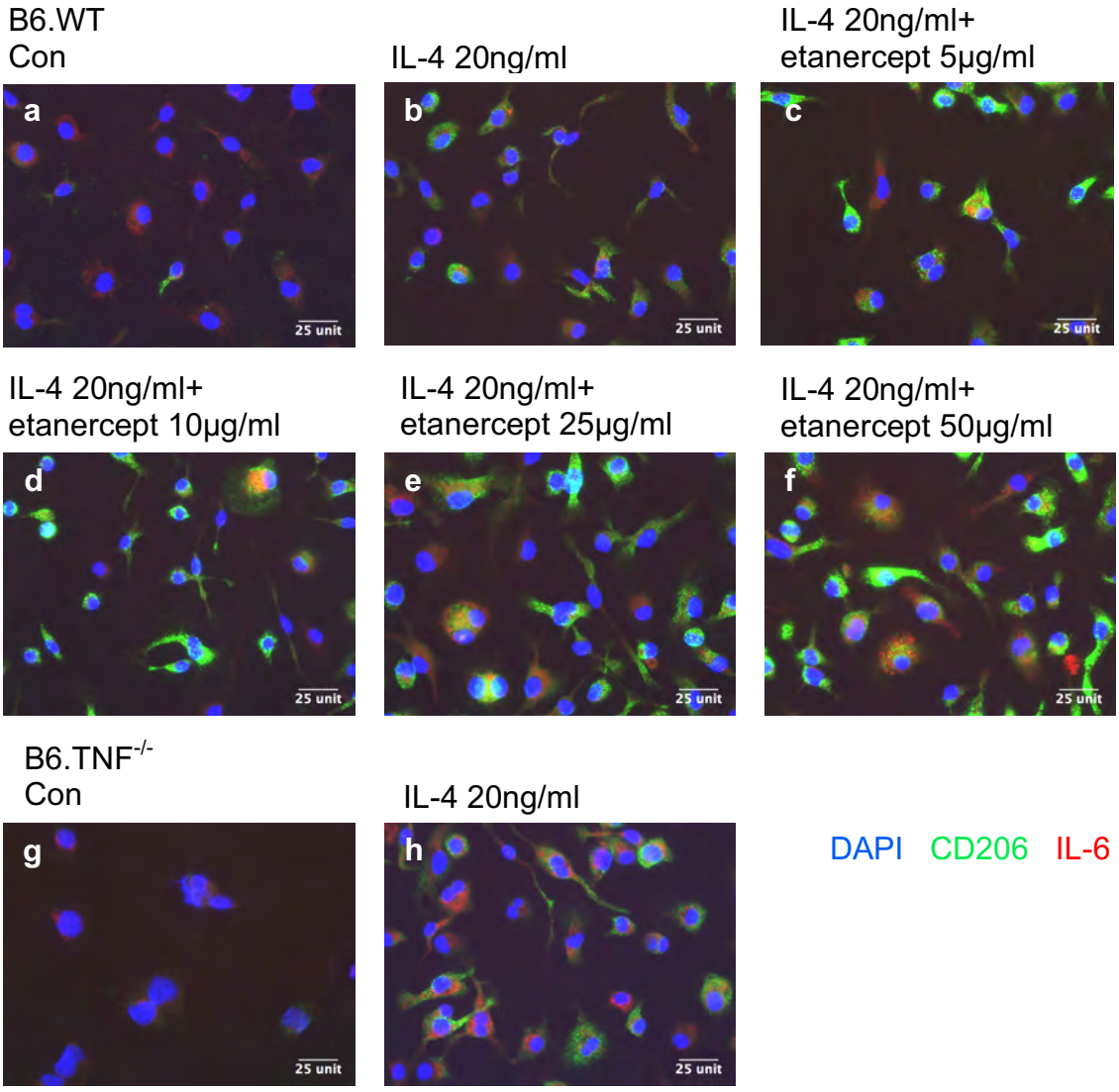


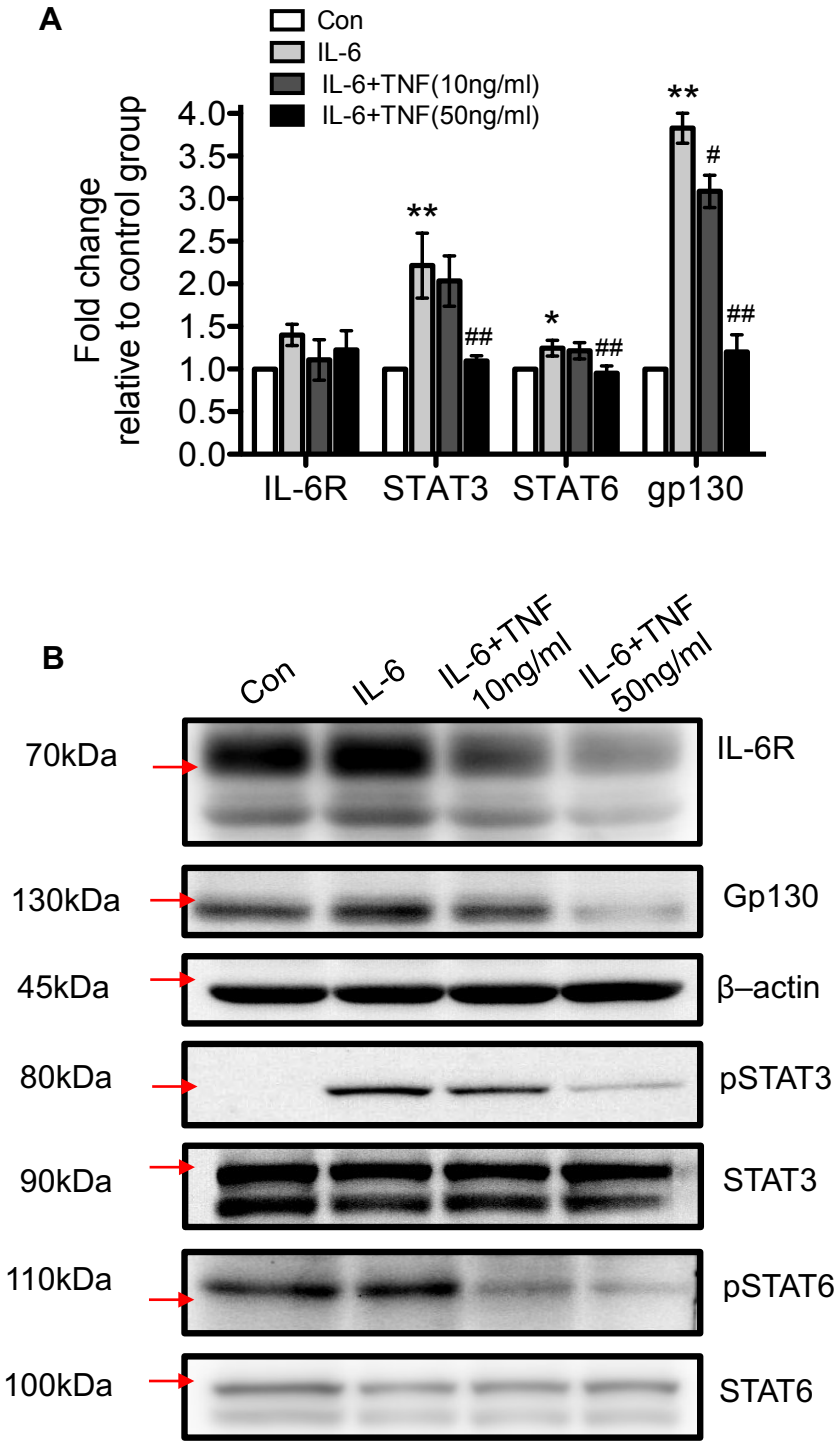
Figure 4.3 The balance of TNF and IL-6 in macrophage differentiation

Bone marrow-derived macrophage of B6.WT were cultured with IL-4 and increasing concentration of etanercept as indicated (a-f). The expression of IL-6 (Red) and CD206 (Green) were monitored by immunofluorescence. Bone marrow-derived macrophage from B6.TNF$^{-/-}$ were incubated with Medium (g) or IL-4 (h) Immunofluorescence photographs represent sections of cells from each culture condition labelled for IL-6 (red), CD206 (green) and nuclei (blue). One of three independent experiment.

4.2.3 The regulatory effect of the balance between TNF and IL-6 affected gp130/STAT3 and IL-4/STAT6 signaling

IL-6 signal transduction requires the formation of a trimer IL-6R complex with signaling transduction via membrane glycoprotein gp130. To clarify the association of IL-6R and gp130 after IL-6 and TNF treatment, we examined each component by western blot and RT-PCR. Exposure to IL-6 upregulated levels of both IL-6Rα and gp130 (Fig 4.4 A&B). Semi-quantification showed IL-6Rα was significantly higher after IL-6 treatment compared to the untreated group (Fig 4.4C-a), while change to gp130 was not significant difference though the level was higher in IL-6-treated group (Fig 4.4C-b). In contrast, TNF treatment inhibited IL-6-induced elevation of IL-6R and gp130, especially at 50ng/ml, with IL-6Rα and TNF almost completely blocked (Fig 4.4), indicating that TNF inhibited IL-6-IL-6Rα /gp130 signaling pathway. Activation of STAT3 is tightly involved in myeloid cell differentiation and maturation states [203, 204], thus we wanted to estimate if STAT3 was affected during TNF and IL-6 interaction. pSTAT3 was not detectable in the control group, but it came up upon IL-6 treatment. However, TNF-inhibited IL-6-induced STAT3 phosphorylation, showing that this process is correlated with IL-6-induced STAT3 activation (Fig 4.4).

IL-4 polarize M2 macrophage through STAT6 activation[205], and impaired STAT6 affected M2 macrophage activation [206]. As IL-6 signaling had an effect on IL-4-medicated STAT6 phosphorylation in myeloid cells [146], we asked whether STAT6 phosphorylation is modulated in our case. In line with pSTAT3, pSTAT6 showed a similar trend in response to either IL-6 or IL-6+TNF, which suggested that TNF inhibited IL-6-enhanced M2 macrophage activation and this process might not only act through down-regulation of STAT3 activation but also the reduction of IL-4-induced STAT6 phosphorylation.


A
Con
IL-6
IL-6+TNF(10ng/ml)
IL-6+TNF(50ng/ml)
Fold change relative to control group
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
**
#
##
##
##
IL-6R
STAT3
STAT6
gp130
B
Con
IL-6
IL-6+TNF 10ng/ml
IL-6+TNF 50ng/ml
70kDa
IL-6R
130kDa
Gp130
45kDa
β–actin
80kDa
pSTAT3
90kDa
STAT3
110kDa
pSTAT6
100kDa
STAT6

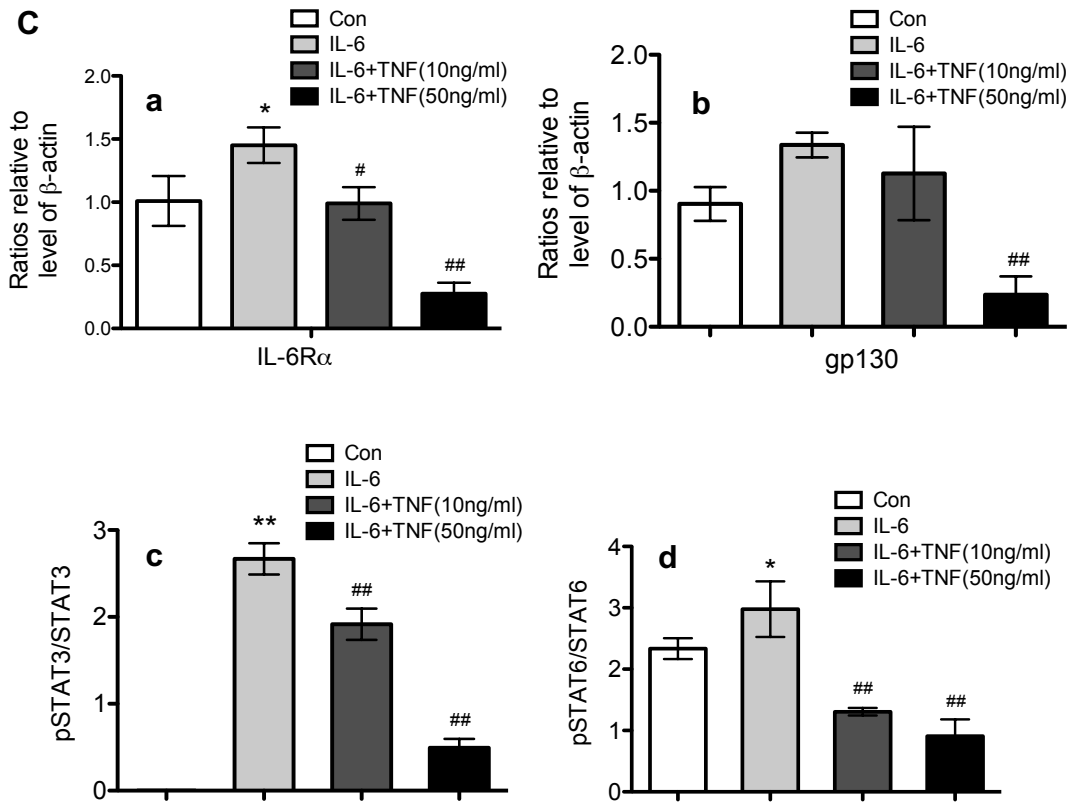


Figure 4.4 The regulatory effect of TNF and IL-6 in macrophage differentiation

(A) A qRT-PCR analysis of different signaling molecules in M2 macrophage treated with IL-4 and TNF. Results were calibrated to the control group (M2) value and represent Means ± SD of three. Statistical analysis was performing using ANOVA with Tukey's pos-test, and the results were considered significant with a *p<0.05, **p<0.01 comparing to control group, and #p<0.05 and ##p<0.01 comparing to IL-6-treated group. (B) Western Blot analysis of different signaling molecules. One of three experiments. (C) and (D) relative changes and the ratio of different signaling molecules. Results were calibrated to the control group value and represent Means ± SD of three. *p<0.05, **p<0.01 comparing to control group, #p<0.05 and ##p<0.01 comparing to IL-6-treated group. One-way ANOVA analysis with Tukey's post-comparison.

4.3 Conclusion and discussion

IL-6, the pro-inflammatory cytokine, has been demonstrated to exhibit anti-inflammatory characteristics in different scenarios. In this chapter, IL-6 was shown to inhibit monocyte differentiation to DCs, thereby potentially reducing pro-inflammatory immune response. IL-6 treatment caused the cells producing less CD11c, while exposure of cells to both IL-6 and TNF together comparatively increased the number of $CD11c^+$ cells. Although IL-6 interfered monocyte differentiation from DCs to macrophage has been reported, it was investigated under co-culture condition with fibroblasts cells [192]. Thus, the results may be affected by the existing effectors in the complex condition. Our result generated from the pure and simple *in vitro* experimental condition overcame the limitations and clearly showed the inhibition role of IL-6, preventing monocyte differentiation to DCs. It has been proven that GM-CSF and IL-4 in combination activated monocytes and potentially caused ectodomain shedding of M-CSFR, inhibiting macrophage differentiation from monocytes [207]. In line with that, we found M-CSFR gene expression increased when the cell was treated with IL-6 and declined on further exposure to TNF, demonstrating IL-6 inhibited monocyte differentiation to DC through up-regulation of M-CSFR expression.

Additionally, it has been reported that IL-6 increased alternative activation of macrophages and limited LPS-induced endotoxemia that prevented the disease development [146]. As IL-6 interfered monocyte differentiation, it may be endowed with the ability to affect macrophage polarization. Thus, we compared the effect of IL-6 on M0, M1 and M2 macrophage. We found M2 macrophages were affected by IL-6 as well as TNF. IL-6 facilitated M2 macrophages polarization with increased Arg-1 and CD206 expression and TNF inhibited the process producing more iNOS.

Overall, the data presented above proved IL-6 exhibits an anti-inflammatory characteristic as it promotes monocytes to differentiate into macrophages rather than DCs, and facilitates M2 macrophages polarization. Furthermore, it also provided additional information about the negative regulatory effect of IL-6 and TNF in monocyte/macrophage differentiation. In order to clarify their interaction, we set out an experiment using etanercept, a fusion protein acting as TNF inhibitor, to block TNF *in vitro*. M2 macrophage had increased IL-6 expression when treated with etanercept, and also displayed increased CD206. Moreover, result of IL-4-treated cells from B6.TNF$^{-/-}$ mice also demonstrated reciprocal inhibition of IL-6 and TNF.

Previously, IL-6 was proven to inhibit TNF production [208], as well as a Th1 response [209]. Also, TNF/IL-6 balance on regulating granulomatous and inflammatory response has been revealed in *Rhodococcus aurantiacus* infection [210]. However, to the best of our knowledge, no previous studies have described the effect of TNF/IL-6 balance on monocyte/macrophage differentiation, and the related mechanism is currently not well understood.

With IL-6 stimulation, we found both gp130 and STAT3 were up-regulated, but decreased in the presence of IL-6 and TNF in M2 macrophages. Gp130-STAT3 signaling plays a key role in IL-6-mediated cell differentiation including monocyte and macrophage differentiation. DC-mediated T cell activation was enhanced in IL-6 KO mice and suppressed in IL-6 transgenic mice [203], indicating the importance of IL-6-STAT3 in DC maturation and activation. STAT3 is required for gp130-induced macrophage differentiation and mutation of gp130 that are unable to help the differentiation from myeloid cells to macrophages [211]. Interestingly, the IL-6/STAT3 pathway was required for chemokine production of macrophages after its

activation, which could promote a forward feed of macrophages [212]., Therefore, our data demonstrated the importance of gp130/STAT3 in macrophage polarization.

Besides gp130, IL-6 exerts its biological processes that require IL-6R to form a complex. Interestingly, IL-6R has two different types, membrane bound IL-6R and soluble IL-6R, the latter one is produced by the enzymatic cleavage of mIL-6R by metalloproteinase or alternative splicing, can also bind with IL-6 and form the complex with gp130. This unique receptor signaling is termed as IL-6 trans-signaling and the previous one is named as IL-6 classical signaling. Although IL-6R seems to be very important in signaling transduction, IL-6R-mediated responses are very limited and restricted mainly to the cells that express mIL-6R, such as hepatocytes, some epithelial cells and some leukocytes [140]. Thus, IL-6 trans-signaling pathway acts as a compensative pathway to trigger an IL-6-mediated response in cells that would not express mIL-6R. As the results shown, the membrane IL-6Rα (90kDa) were up-regulated upon IL-6 treatment and decreased in the presence of TNF and IL-6. Indeed, we observed more than one IL-6R bands (lower than 90kDa, data now shown) in the experiment. Although their molecular mass matched to the description of sIL-6R in literature [187], whether they are generated by alternative splicing or proteolytic cleavage need to be validated by using specific antibody. IL-6 classical signaling pathway was tightly associated with anti-inflammatory response, while IL-6 trans-signaling is more likely involved in pro-inflammatory response [186]. Therefore, our results illustrated IL-6 played an anti-inflammatory role in monocyte/macrophage differentiation. Even though the bands possibly represent sIL-6R were observed, IL-6 classical signaling may outweigh IL-6 trans-signaling to skew the immune response.

IL-4-STAT6 axis is a vital inducer of M2 macrophage polarization and impaired of either IL-4Ra or STAT6 inhibited M2 macrophage activation [213, 214]. Interestingly, IL-4 signaling was reported to have interaction with IL-6 signaling. Myeloid effector cells were found upregulate IL-4R expression during inflammatory condition, which depended on IL-6 production [215]. IL-6 induced IL-4-receptor expression and augmented the response to IL-4 in macrophages in a cell-autonomous manner, and IL6R$\alpha^{\Delta myel}$ mice were resistant to IL-4-mediated alternative macrophage polarization and exhibited increased susceptibility to LPS-induced endotoxemia [146]. Therefore, whether negative regulation between TNF and IL-6 also affected macrophage for IL-4-dependent activation of STAT6 is worth determining. We found the level of pSTAT6 increased upon IL-6 stimulation and was mostly inhibited by extra TNF, which revealed not only STAT3 but also STAT6 signaling contributes to IL-6-facilitated M2 macrophage polarization. Follow up studies on this work could lead to interesting insights regarding the regulatory effect of TNF and IL-6 *in vivo*.

In summary, our results here proved IL-6 has the ability to interfere the process of monocytes differentiation and macrophage polarization and revealed the importance of the regulatory balance between IL-6 and TNF in this case. These results were consistent with our previous finding *in vivo* that IL-6 elevated in an alternative activated macrophage population from B6.TNF$^{-/-}$ mice upon *L. major* infection, provided a potent explanation for how IL-6 caused the defect of the immune response in the absence of TNF, and set the new basis to identify the interaction of TNF and IL-6 signaling in macrophages in conditions where alternative macrophage activation is also of critical importance in leishmaniasis.

Chapter 5.
Lack of TNF modulates splenic DC populations during *L. major* infection

5.1 Introduction

The spleen is a secondary lymphoid organ, and its well-organized structure of phagocytic cells in combination with a lymphoid compartment enables it to act both as a filter for blood-borne exogenous pathogens as well as initiate anti-pathogen immune reactivity.

The spleen consists of two main compartments, the red pulp and the white pulp, which are vastly different in morphology and function. Venous sinuses and splenic cords compose the red pulp, which is essential for blood filtering and iron recycling by red pulp macrophages. In contrast, white pulp closely resembles the structure of a lymph node, which is made of greyish areas of lymphoid tissue, surrounding a central arteriole to form PALS, B cells follicles, and marginal zone, and contributes to foreign material removing [216]. The inner layer of PALS contains a large number of $CD4^+$ T cells and $CD8^+$ T cells, interdigitating DC and migrating B cells, while the outer layer mainly consists of macrophages. As a contiguous area of PALS, the B cell follicles consist of B cells, $CD4^+$ cells and follicular DCs. The marginal zone is located at the interface of the red pulp and PALS, and is for filtering antigens as well as pathogens and play an important role in antigen processing. It contains a large number of resident cells, especially splenic macrophages and DCs, which participate in the innate immune response to microorganisms as well as in mounting an adaptive response against blood-borne antigens.

As we mentioned previously, *L. major* infection only caused skin lesions in B6.WT mice. However, it is able to induce visceralization of leishmaniasis in the absence of TNF, that is the similar symptoms observed in *L. donovani*-induced visceral leishmaniasis. In *L. donovani*-induced visceral leishmaniasis, the spleen is also a target associated with the persistence of parasite infection and remodelling of the lymphoid structure. There is a profound dysregulation

of the immune response and different types of cells involved in the disease progression. Macrophages in the marginal zone are the major phagocytic cell population for parasite clearance in the early stages of visceral leishmaniasis. It had been reported that more than 90% of parasites are phagocytized by splenic macrophages, and 50% of parasites are killed by macrophages in marginal zone within the first 24 hours after infection [217]. This effect has been shown to be dependent on interferon regulatory factor-7, which is the molecule also crucial for sustaining TNF production in the macrophages [218].

In addition to macrophages, DCs are also able to directly phagocytose parasites, but they normally acquire antigen from infected macrophages in the marginal zone. In addition, DC-production of IL-12 is important for the activation of effector T cells, which are recognized as the most important immune cells in the spleen for clearance of infection[155]. IL-12-producing DCs were found in T cell zone and expanded during leishmaniasis [219]. In general, DCs are categorized into pDCs, cDCs and inflammatory DCs when inflammation occurs. cDCs are the main DC population and are strategically located around body barriers and organ entry ports, such as the splenic marginal zone. They can be divided further into $CD8^+$ and $CD11b^+CD8^-$ subpopulations. $CD8^+$ cDCs are resident in non-lymphoid tissue but can migrate to the draining lymph nodes when they are mature, and where they can prime and active the specific $CD8^+$ T cell response to intracellular pathogen [220]. In contrast, $CD11b^+CD8^-$ cDCs are located in both lymphoid and non-lymphoid organs, and exhibit high capacity for $CD4^+$ T cell activation and humoral immunity promotion [221]. pDCs are resident cells derived from bone marrow and comprise around 0.3-0.5% of the cells in lymphoid tissue through the blood circulation [222]. Plasmacytoid DCs exhibit a low capacity for antigen presentation having a low level of both MHC class II and costimulatory molecule expression in the steady state. However, they

produce large amounts of type I IFN and their capacity to present foreign antigens is upregulated upon stimulation [223]. pDCs can be easily separated from cDCs by their high-expression of B220, and are mainly distinguished by the markers $B220^{+}CD11b^{-}CD11c^{+}MHCII^{lo}Ly6C^{-}$. pDCs have been shown to efficiently prime the $CD4^{+}$ T cell response in the lymph nodes [224], induce Th1 polarization [225, 226], and prime [227] and cross-prime $CD8^{+}$ T cell responses [228, 229]. Monocyte-derived DCs are increased greatly at the site of infection, where they produce IL-12 and initiate a pathogen-specific T cell response, that is also essential for T cell immunity against pathogens.

Besides the innate cells, the aberrant T and B cell immune responses are also involved in spleen infection during leishmaniasis. B cell activation is found in visceral leishmaniasis, and mice lacking B cells showed enhanced resistance to parasite infection [230].

Chronic leishmaniasis leads to immune exhaustion including the exhaustion of $CD4^{+}$ and $CD8^{+}$ T cells, decreased of IFN-γ and TNF, and elevated inhibitory molecules such as programmed cell death protein-1 (PD-1) and IL-10 during visceral leishmaniasis. Blocking interaction between PD-1 and PD-1 ligand restored T cell function and reduced parasite burden [231]. Similarly, increased IL-10 production can be found in the chronic phase of infection, affecting antigen processing cell maturation, inducing their death, and consequently impeding T cell function [232]. IL-10 neutralization during experimental visceral leishmaniasis improved $CD4^{+}$ T cell responses and led to disease resolution [233].

Therefore, the immune response of spleen against leishmaniasis is complex and requires not only innate immunity but also adaptive immunity to be effective. As we mentioned before, *L.*

major only induced cutaneous infection in resistant B6.WT mice. Although spleen infection during cutaneous leishmaniasis has been observed when B6.WT mice with deleted TNF gene, there has been no formal investigation and explanation for this phenomenon. Therefore, it is worth exploring why the lack of the TNF gene leads to splenic infection. Understanding differences of the immune response in the spleen against *L. major*-induced leishmaniasis in the absence of TNF and determining the underlying mechanism that may contribute to disease resolution.

5.2 Results

5.2.1 Spleen infection was observed in *L. major*-induced cutaneous leishmaniasis in B6.TNF$^{-/-}$ mice

L. major does not induce visceral leishmaniasis in B6.WT mice, but we observed visceral infection in *L. major*-infected B6.TNF$^{-/-}$ mice. Before infection with *L. major*, the appearance of the spleens from B6.WT and B6.TNF$^{-/-}$ mice were similar to each other (Fig 5.1A, upper panel). However, spleens from B6.TNF$^{-/-}$ mice were extensively enlarged, around 2-3 times larger than that from B6.WT mice (Fig 5.1A, bottom panel). Also, the color of the spleens was comparatively dim in B6.TNF$^{-/-}$ mice (Fig 5.1A, bottom panel).

Prior to infection, spleens of B6.WT and B6.TNF$^{-/-}$ mice were around 100mg, and there was no significant difference in spleen weight between groups (Fig 5.1A). At day 28 p.i., spleen weight increased sharply in both groups (Fig 5.1B). Although the weight of spleen from B6.TNF$^{-/-}$ mice was comparatively higher, it did not show a significant difference to that of B6.WT mice. From day 28 p.i., the weight of the spleen in B6.TNF$^{-/-}$ mice was consistently larger than B6.WT mice, reaching over 300mg at day 42 sacrifice (Fig 5.1A). In contrast, the weight of spleen in B6.WT mice returned to pre-infection level after day 28 p.i. (Fig 5.1B).

Enlarged spleen is a typical hallmark of visceral leishmaniasis caused by *L. donovani*. Whether enlarged spleens is caused by *L. major* infection needs to be determined. In order to confirm the parasite infection, the parasite load was evaluated from day 21 p.i.. In keeping with the increased spleen weight, a large number of parasites were found in B6. TNF$^{-/-}$ mice, reaching

6000 per gram at day 21 p.i., and increasing steadily up to 150000 per gram at day 42 p.i.. In contrast, parasites were rarely found in the spleen of B6.WT mice during infection (Fig 5.1C).

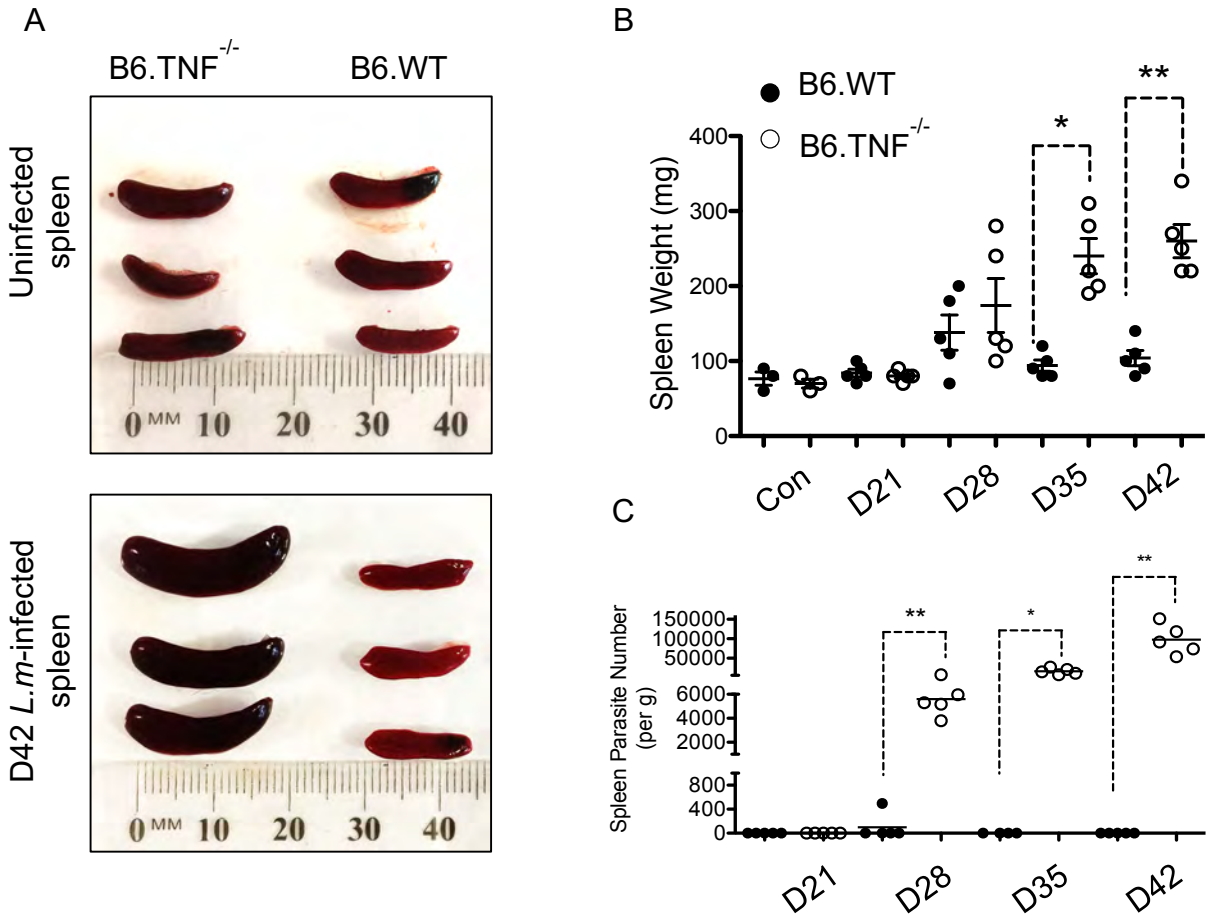


Figure 5.1 The infection of spleen in B6.WT and B6.TNF$^{-/-}$ mice

(A) The appearance of uninfected and *L. major*-infected (day 42) spleens from B6.WT and B6.TNF$^{-/-}$ mice. There was no difference in spleen appearance between B6.WT and B6.TNF$^{-/-}$ mice without infection, while the size of spleen of B6.TNF$^{-/-}$ mice increased greatly at 42 day p.i., compared to B6.WT mice. (B) The weight of spleens from B6.WT and B6.TNF$^{-/-}$ mice was determined during *L. major* infection. Five B6.WT and B6.TNF$^{-/-}$ mice were used to determine the spleen weight for each time point. (C) The number of viable parasites in the spleen of B6.WT and B6.TNF$^{-/-}$ mice was determined by limiting dilution analysis. The mean parasitic burden in the splenic tissue of five mice is shown. One circle represents one animal. All data are represented as mean ± SD. Significance was calculated using a two tailed Mann-Whitney U test (*p<0.05, **p<0.01).

5.2.2 Analysis of the splenic microarchitecture in B6.WT and B6.TNF$^{-/-}$ mice during leishmaniasis

TNF was reported to regulate the fine architectural organization of lymphoid tissue in the late stage of embryonic development. Absence of TNF led to disruption of the spleen microenvironment including a less demarcated T cell and B cell zone and absence of germinal centres after immunization [42]. After day 42 p.i., the structure of spleen in B6.WT mice was still well-organized. The T cells and B cells zones were clearly separated, and the germinal centre and marginal zone were still visible. However, these areas were poorly demarcated and organized in B6.TNF$^{-/-}$ mice at day 42 p.i.. A large number of lymphoid cells were dispersed, and white and red pulps were not detected (Fig 5.2).

Inoculation *L. major* into the footpads of BALB/c mice leads to lethal systemic infection. The immune response fails to protect the animal from the metastatic spread of parasites to the visceral organs, which is similar to the cause of infection these present in *L. major*-infected B6.TNF$^{-/-}$ animals. Therefore, we include BALB/c mice as a positive control, and compare it against B6.TNF$^{-/-}$ mice. BALB/c mice showed no visible difference of the splenic structure compared to that of the other two strains before infection, but after challenge with *L. major*, splenic white and red pulp were clearly separated and the boundaries between white pulps were contiguous and indistinguishable. Although the structure became less demarcated, it still has the basic impact functional area.

Correspondingly, there was no significant difference of T cell percentage including CD4^{+} and CD8^{+} T cells at day 42 p.i (Fig 5.3A, E & F). Based on CD3^{+} cells, the relative percentage of CD4^{+} and CD8^{+} T cells was not changed in the two mouse strains, which is in line with the

results acquired from infected BALB/c mice (Fig 5.3B). Moreover, B cell population showed no significantly change based on $CD3^{-}CD19^{+}$ analysis (Fi 5.3C & G), although the germinal centre was missing in B6.$TNF^{-/-}$ mice.

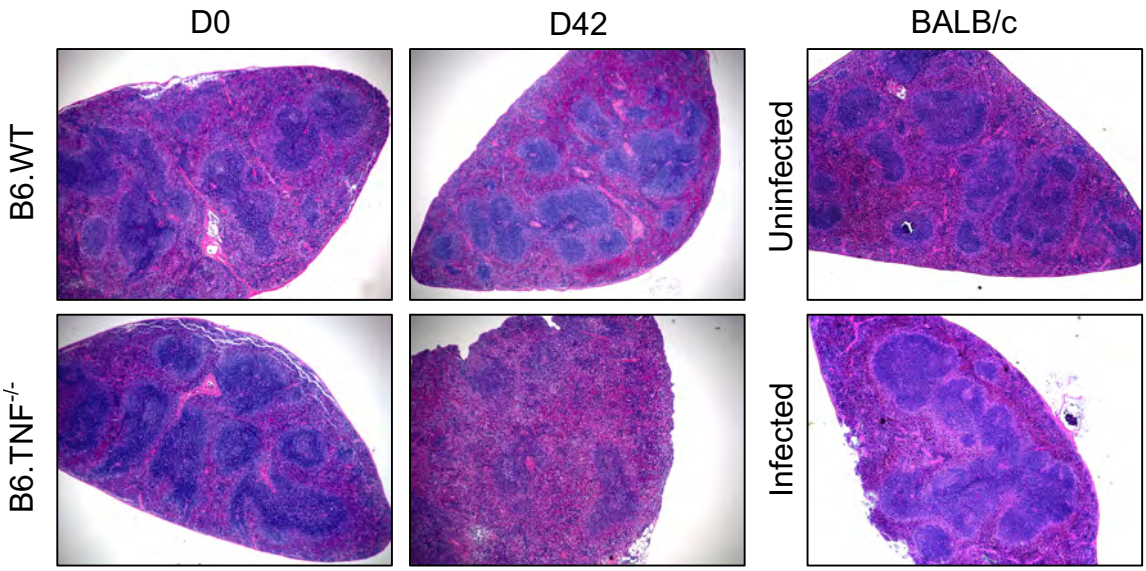
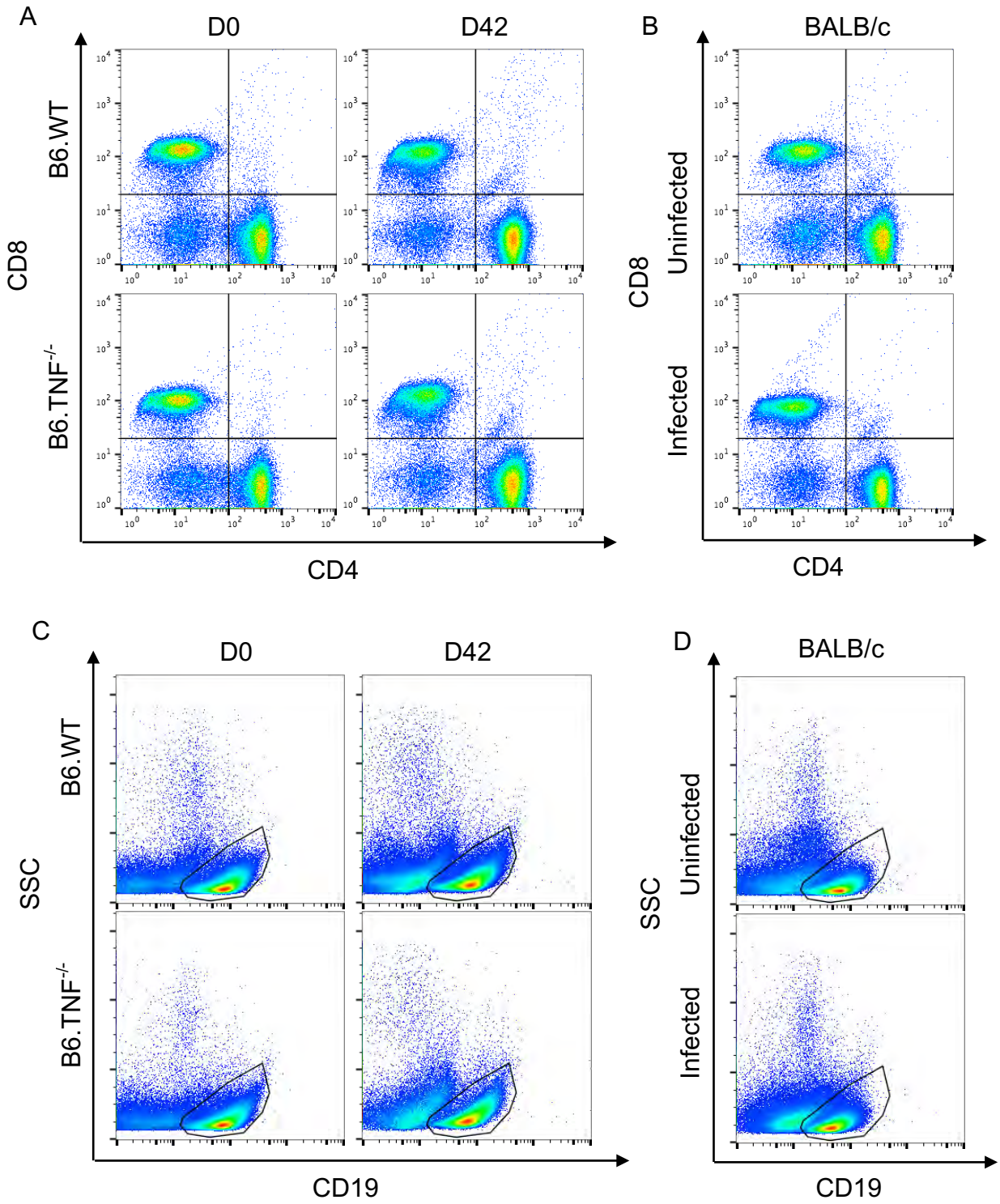


Figure 5.2 H&E staining of spleen tissue from B6.WT and B6.$TNF^{-/-}$ mice

Representative H&E stained spleen section at low (5 x) are shown (N=5 per group). There was no visible difference of spleen architecture between B6.WT and B6.$TNF^{-/-}$ mice before infection (left panel). After day 42 p.i. (middle panel), a less organized spleen structure was observed in B6.$TNF^{-/-}$ mice. Spleen was lager, white and red pulp were hardly detected and huge numbers of cells were dispersed. Uninfected and infected spleen from BALB/c mice are shown on the left panel, which are the positive control for B6.WT and B6.$TNF^{-/-}$ mice.


A
D0
D42
B6.WT
B6.TNF-/-
CD8
CD4
B
BALB/c
Uninfected
Infected
CD8
CD4
C
D0
D42
B6.WT
B6.TNF-/-
SSC
CD19
D
BALB/c
Uninfected
Infected
SSC
CD19

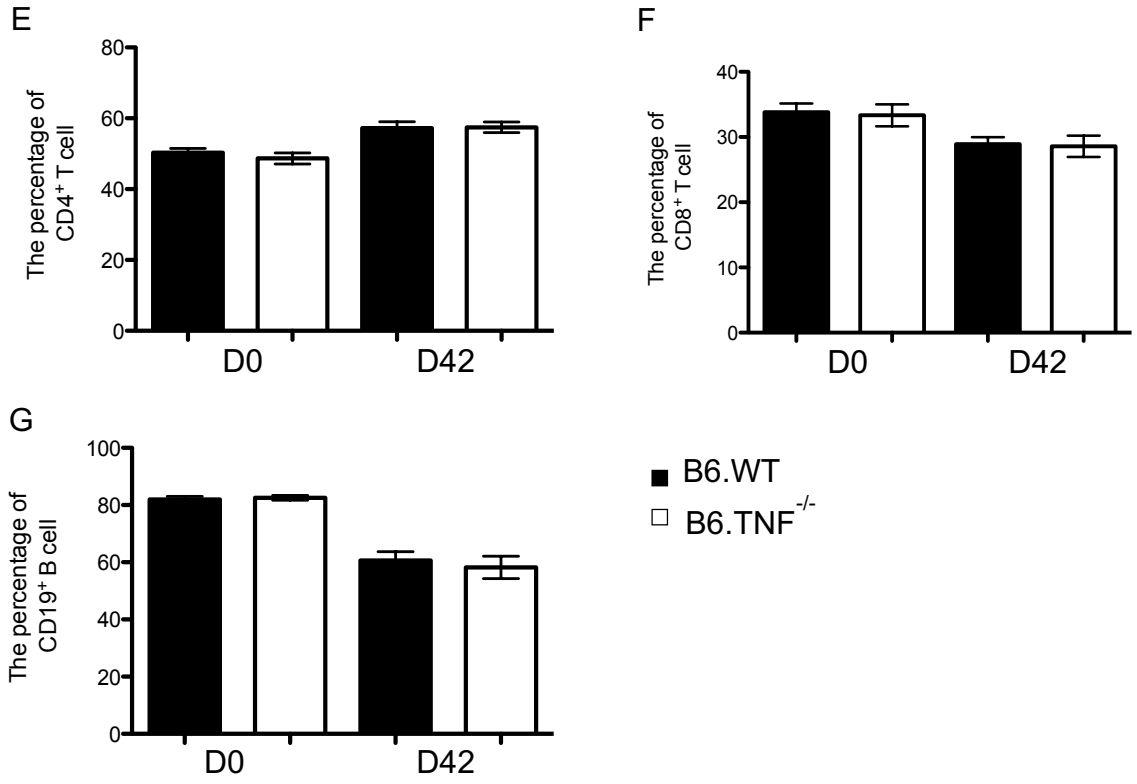


Figure 5.3 T cell and B cell population found in B6.WT and B6.TNF$^{-/-}$ mice.

Flow cytometric analysis of the population of CD4^{+}, CD8^{+} T cells and CD19^{+} B cells from B6.WT, B6.TNF$^{-/-}$ and BALB/c mice in the course of *L. major* infection (A-D). Although B6.TNF$^{-/-}$ mice showed severe spleen infection, none of the population proportions showed a significant difference in comparison to B6.WT mice during leishmaniasis (E-G). Five B6.WT and B6.TNF$^{-/-}$ mice were used for each time point. Error bars represent the mean ± SD from three independent experiments.

5.2.3 Splenic CD11b$^+$ cells in B6.TNF$^{-/-}$ mice are reduced during leishmaniasis

Although there was no alteration of T cell and B cell proportions between B6.WT and B6.TNF$^{-/-}$ mice during infection, changes of mononuclear phagocytes including macrophages, DCs and monocytes in the absence of TNF during spleen infection have yet to be determined. Splenic macrophages are the main phagocytic cell population responsible for parasite clearance and have been found to kill more than 50% of the initial parasite inoculum in the early visceral leishmaniasis [18]. Here, we found the number of CD11b$^+$ cells was comparable in B6.WT and B6.TNF$^{-/-}$ mice prior to infection, but sharply decreased in the B6.TNF$^{-/-}$ at day 42 p.i. compared to B6.WT mice (Fig 5.4A). *L. major*-susceptible BALB/c mice displayed similar results where CD11b expression was higher without infection than post-infection (Fig 5.4A). Interestingly, F4/80$^+$ cell population of CD11b$^+$ cells was significantly higher in B6.TNF$^{-/-}$ mice at day 42 p.i. compared to B6.WT mice (Fig 5.5A & C), which was also in line with the results found in BALB/c mice (Fig 5.5B), indicating B6.TNF$^{-/-}$ mice displayed a similar immune response to susceptible BALB/c mice upon *L. major* infection. Additionally, F4/80$^+$ cell population showed very low expression of CD11b and did not express Ly6C and CCR2 (data not shown here), which implied this population exhibited the characteristics of resident macrophage rather than migrated monocyte-derived macrophages and may become the shelter of parasites [234].

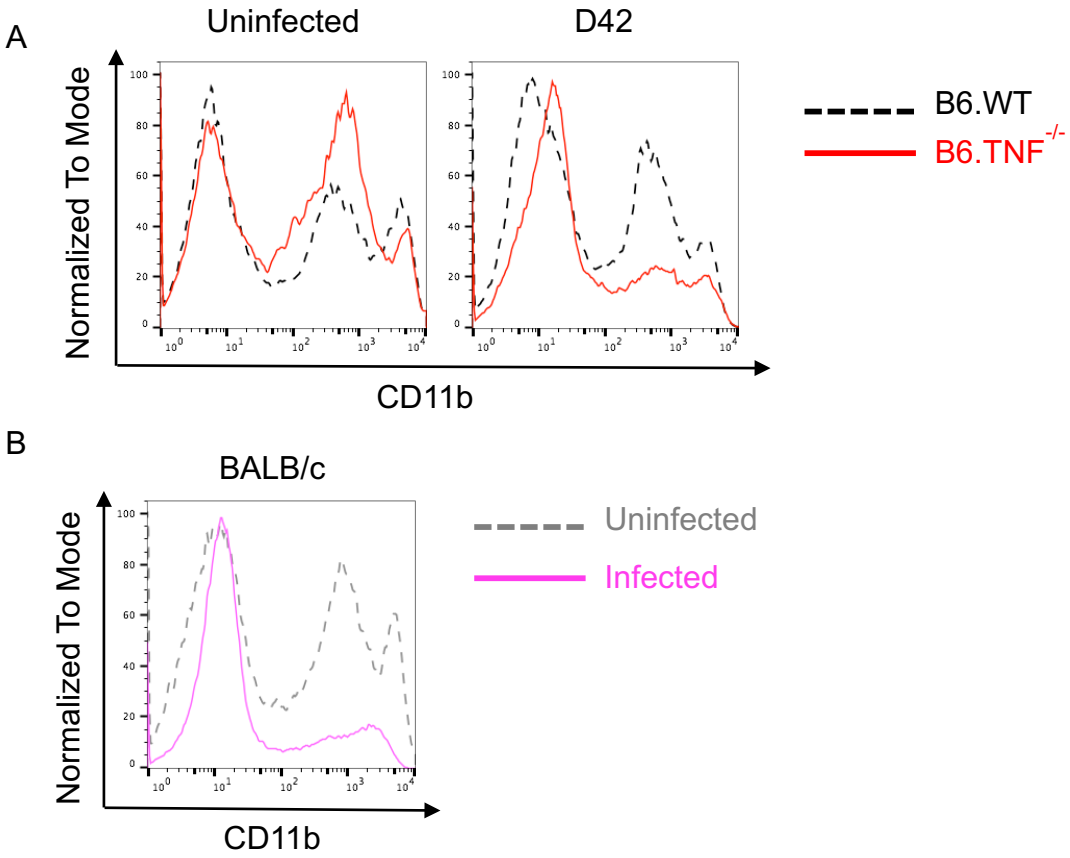


Figure 5.4 CD11b^{+} cell population in B6.WT and B6.TNF$^{-/-}$ mice

(A) Splenic CD11b^{+} cell population from uninfected and *L. major*-infected B6.WT and B6.TNF$^{-/-}$ mice (day 42). Black dot line represented B6.WT mice and red line represented B6.TNF$^{-/-}$ mice (n=5) (B) Splenic CD11b^{+} cell population in uninfected and *L. major*-infected BALB/c mice. The grey dotted line represents uninfected BALB/c mice and the pink line represents infected BALB/c mice (n=3).

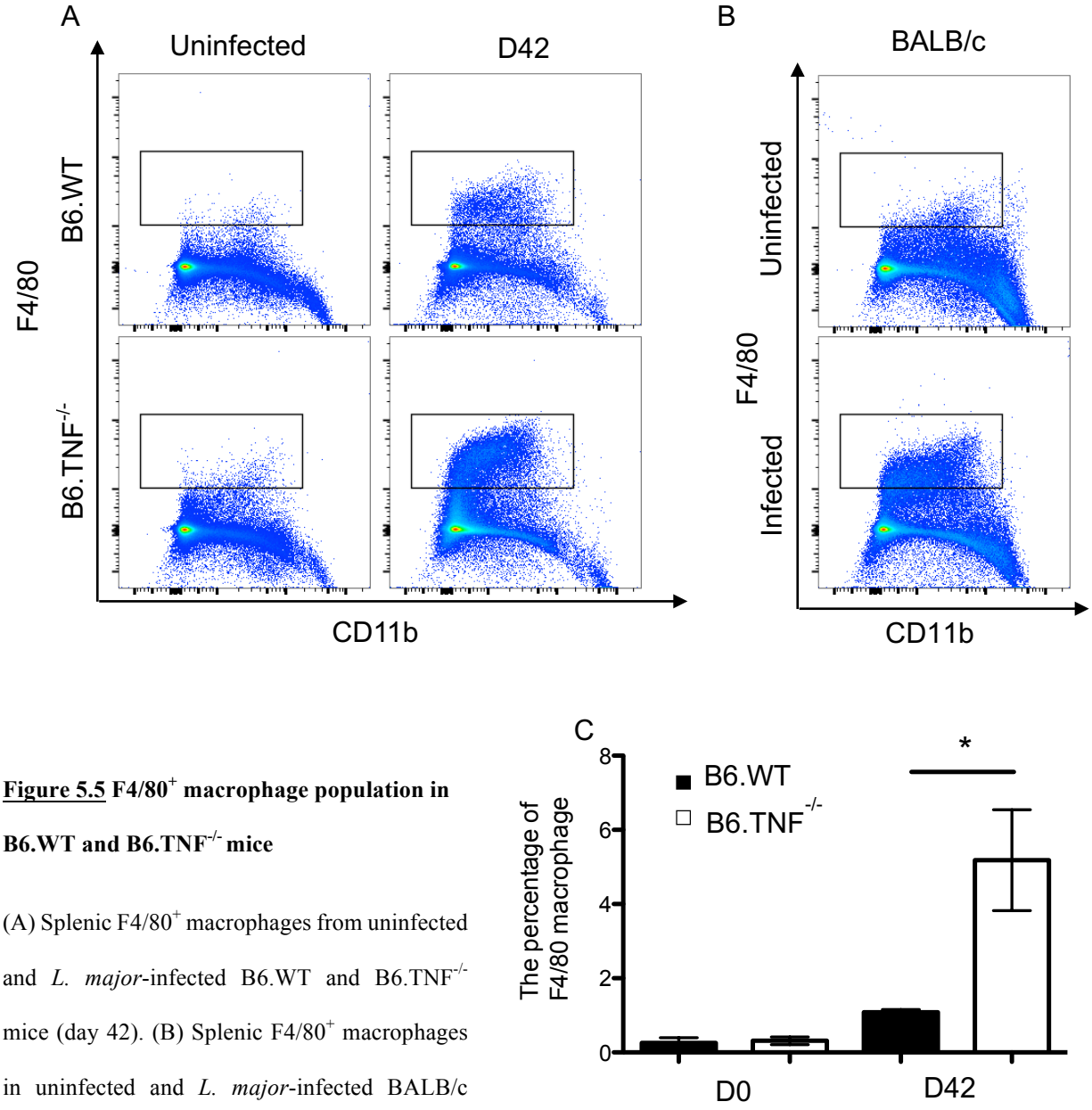


Figure 5.5 F4/80$^+$ macrophage population in B6.WT and B6.TNF$^{-/-}$ mice

(A) Splenic F4/80$^+$ macrophages from uninfected and *L. major*-infected B6.WT and B6.TNF$^{-/-}$ mice (day 42). (B) Splenic F4/80$^+$ macrophages in uninfected and *L. major*-infected BALB/c mice. (C) F4/80$^+$ cells increased significantly in B6.TNF$^{-/-}$ mice compared to B6.WT mice although they showed severe spleen infection. Five B6.WT and B6.TNF$^{-/-}$ mice were used each time point. Error bars represent the mean ± SD from three independent experiments. The *p* values were calculated using a two tailed Mann-Whitney U test (*p<0.05).

5.2.4 Decreased DCs population may lead to visceral infection of spleen in the absence of TNF

Acute immune responses in the spleen plays a key role in controlling *L. donovani* infection during early infection. Rather than macrophages, DCs are crucial for effective immunity [34]. As such, it is worth to investigate whether lack of TNF affect splenic populations of DCs. Firstly, in order to avoid interference from other cell types, 2×10^7 spleen cells were stained with TCR, CD19, NK1.1 and Ly6G to exclude T cells, B cells, NK cells and neutrophils. Non-staining cells were collected and the purity confirmed by flow cytometry. Common DCs subsets in murine spleen can be divided into pDCs and cDCs. These two population can be distinguished based by B220 expression (Fig 5.6A), which is highly expressed on pDCs and is absent on cDCs.

As such, three populations were further identified in $B220^+$ DC population based on CD11c and Ly6C expression, and their characterization was re-analyzed by back-gating (Fig. 5.6C) First, splenic pDCs were defined as $B220^{hi}CD11b^-CD11c^{med}Ly6C^{hi}$ and were significantly decreased in B6.$TNF^{-/-}$ mice compared to B6.WT at 42 day p.i. (Fig 5.7A & E). Moreover, these splenic pDCs showed similarly a low level of MHCII expression in B6.WT and B6.$TNF^{-/-}$ mice when they were normalized to mode, which was in line with the literature shown that pDCs exhibit a low antigen presenting capacity (Fig 5.7B). In addition to pDCs, another two populations were also observed. One expressed $CD11b^{med}CD11c^{hi}$ $B220^+$ but not Ly6C (Fig 5.7A), and its number were significant less in B6.$TNF^{-/-}$ mice with low level of MHCII expression compared to B6.WT littermates (Fig 5.7C). Although the marker expression of this novel population was quite similar to monocyte-derived DCs [235], it was also high expressing B220 and has not been reported previously. Its function requires further investigation. A third

population is $CD11c^{-}Ly6C^{hi}B220^{+}CD11b^{-}$(Fig 3.6B&C, 3.7A) and the number of this population was not has not significantly different between B6.WT and B6.$TNF^{-/-}$ mice.

Conventional DCs is a population which was quite different to pDCs. They do not express B220 but do express MHCII, therefore they are likely to be more proficient at antigen presentation and priming of T cell activation [222]. In spleen, cDCs are normally located in the marginal zone in the steady state, and migrate to T cell zone once inflammation occurs. Here, we found distinctive $CD8^{+}$ and $CD11b^{+}$ cDCs populations in both B6.WT and B6.$TNF^{-/-}$ mice. DCs, once mature, are characterized by high expression of CD11c [175]. Mice of both genotypes showed high expression of CD11c on $CD8^{+}$ and $CD11b^{+}$ cDCs (Fig 5.8B), but CD11c expression was higher in B6.WT rather than B6.$TNF^{-/-}$ mice. Of note, the expression of MHCII of $CD11b^{+}$ cDCs was found particularly to be increased in B6.WT mice (Fig 5.8 A & D), indicating the normal cDCs function in spleen was impaired in the absence of TNF during cutaneous leishmaniasis.

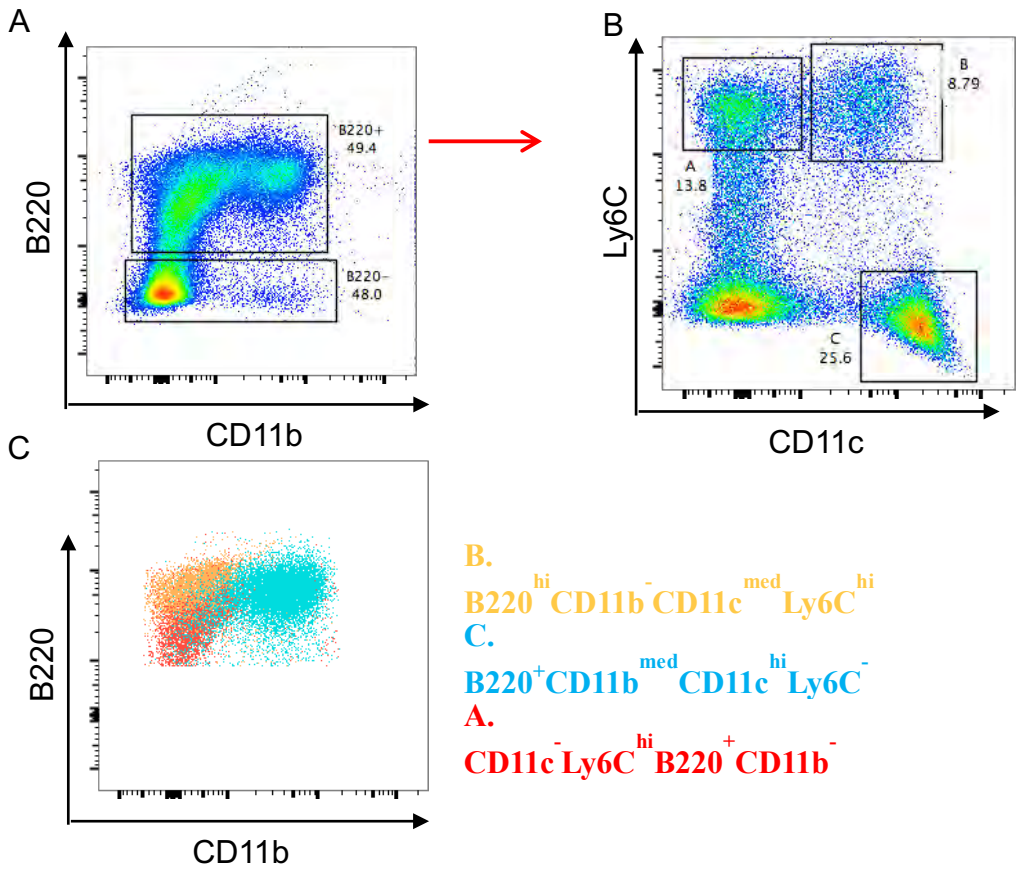
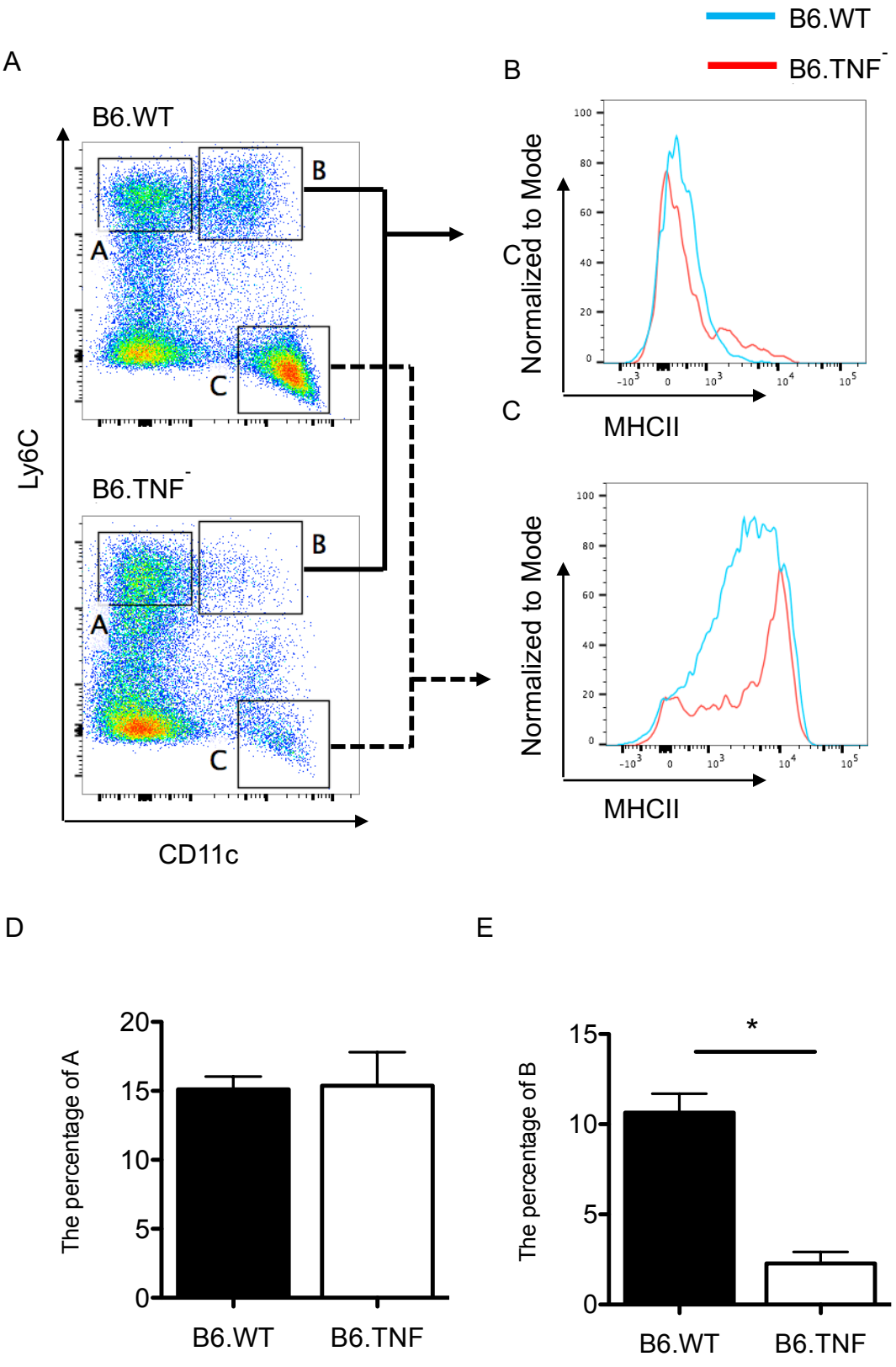


Figure 5.6 Gating strategy for different pDCs population in the spleen

Representative images of different DCs population in WT mice. pDCs highly expressed B220 (A) and further identified based on CD11c and Ly6C (B). Three different populations were presented as A: $CD11c^{-}Ly6C^{hi}B220^{+}CD11b^{-}$, B: $B220^{hi}CD11b^{-}CD11c^{med}Ly6C^{hi}$, and C: $B220^{+}CD11b^{med}CD11c^{hi}Ly6C^{-}$ according to the back-gating (C).


A
B6.WT
B6.TNF-
Ly6C
CD11c
A
B
C
B
B6.WT
B6.TNF-
Normalized to Mode
MHCII
C
D
The percentage of A
B6.WT
B6.TNF
E
The percentage of B
*
B6.WT
B6.TNF

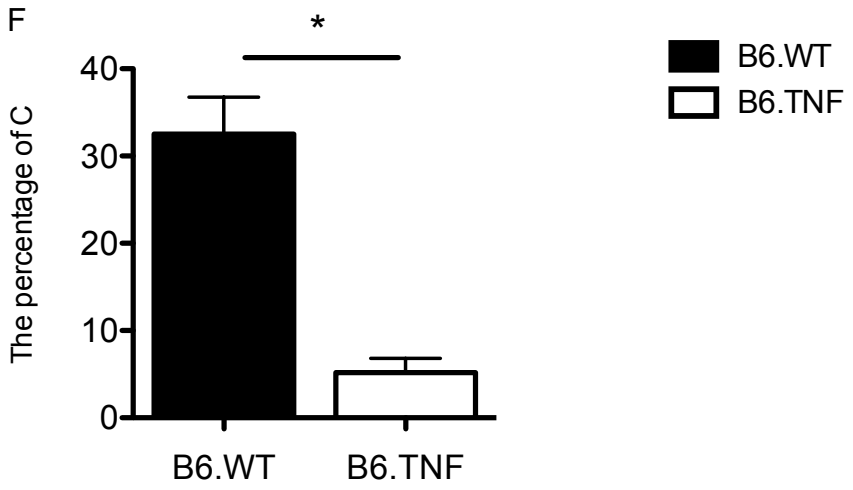
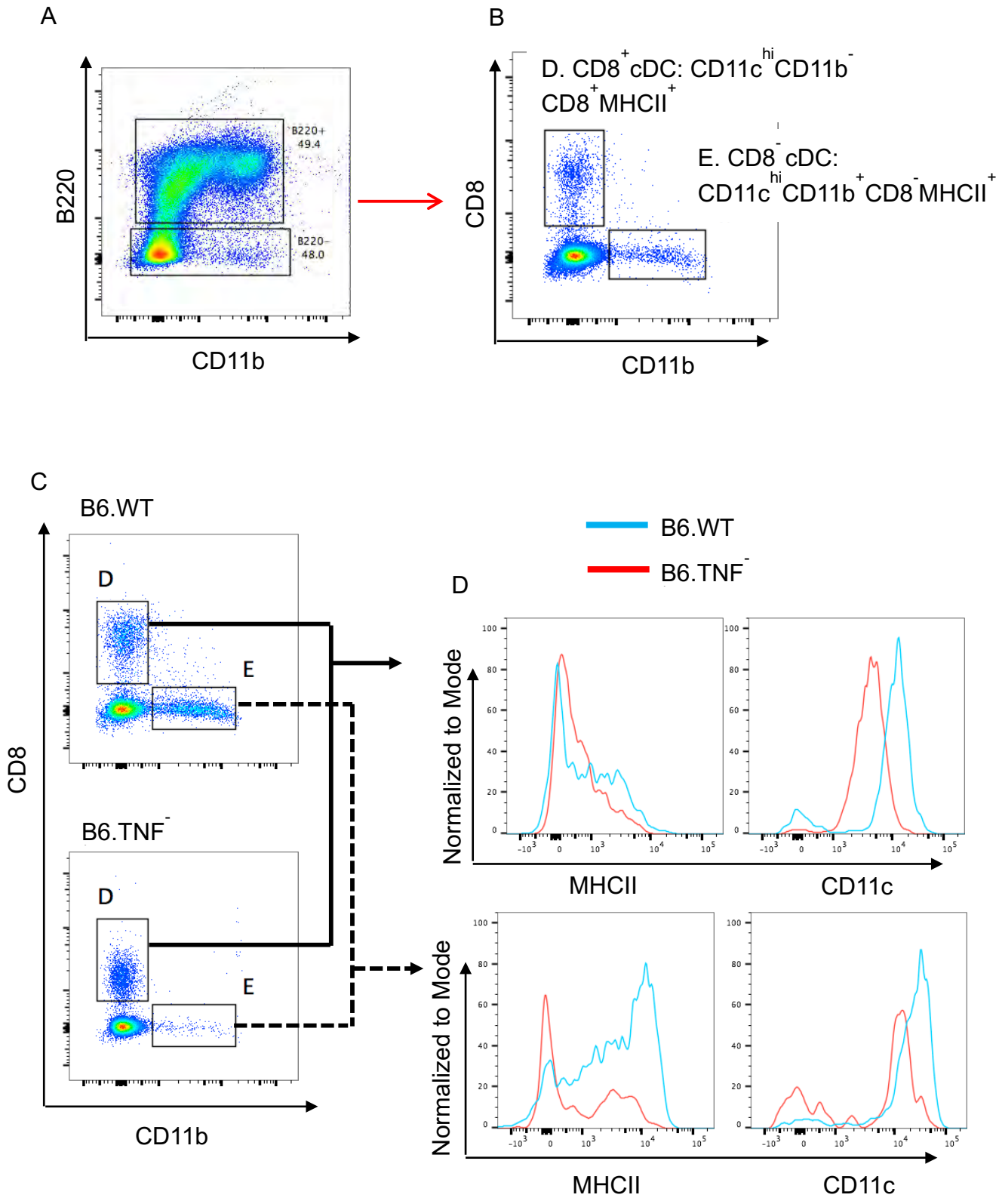


Figure 5.7 Splenic pDCs and B220^{+}CD11b^{med}CD11c^{hi}Ly6C^{-} were fewer in B6.TNF$^{-/-}$ mice

Flow cytometry analysis revealed the changes of three different B220^{+} populations in the spleen from B6.WT and B6.TNF$^{-/-}$ mice at day 42 p.i. of *L .major* infection. CD11c^{-}Ly6C^{hi}B220^{+}CD11b^{-}(population A) did not show any different between two stains (A & D). pDCs (population B) and B220^{+}CD11b^{med}CD11c^{hi}Ly6C^{-} (population C) were significantly fewer in B6.TNF$^{-/-}$ mice in the course of *L. major* infection (A, E & F). Although MHCII expression is comparable in B6.WT and B6.TNF$^{-/-}$ mice, levels decreased in the Mo-pDCs population of B6.TNF$^{-/-}$ mice. Five B6.WT and B6.TNF$^{-/-}$ mice were used each time point. Error bars represent the mean ± SEM from three independent experiments. The *p* values were calculated using a two tailed Mann-Whitney U test (*p<0.05).


A
B220
CD11b
B220+
49.4
B220-
48.0
B
CD8
CD11b
D. CD8+ cDC: CD11c hi CD11b-
CD8+ MHCII+
E. CD8- cDC:
CD11c hi CD11b+ CD8- MHCII+
C
B6.WT
D
E
B6.TNF-
D
E
CD8
CD11b
B6.WT
B6.TNF-
D
Normalized to Mode
MHCII
CD11c
Normalized to Mode
MHCII
CD11c

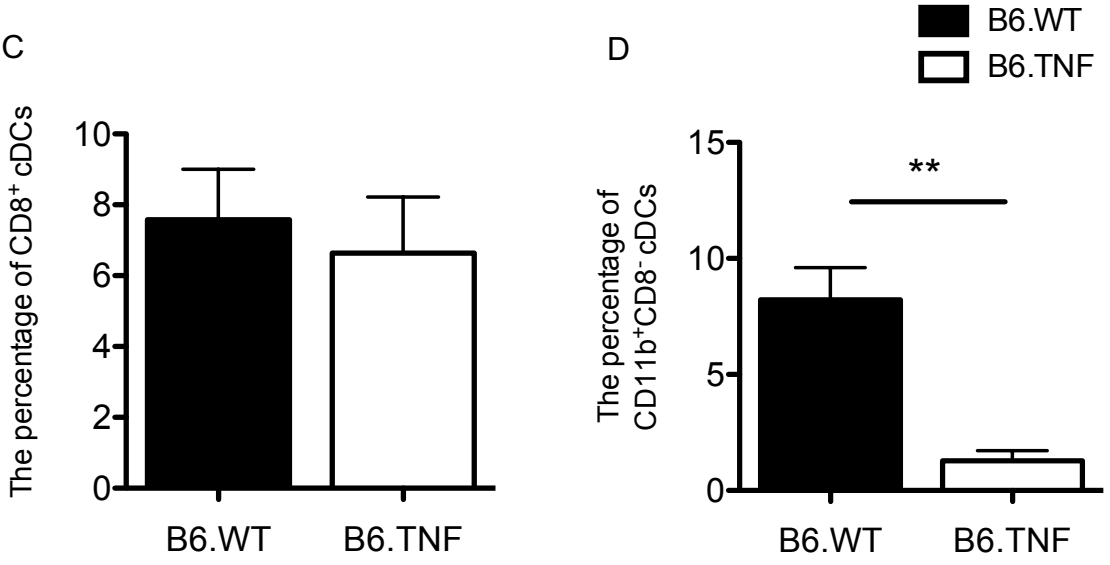


Figure 5.8 Splenic CD8$^-$CD11b$^+$ cDCs and Mo-pDCs were fewer in B6.TNF$^{-/-}$ mice.

Flow cytometry analysis revealed the changes of two different cDCs population in the spleen from B6.WT and B6.TNF$^{-/-}$ mice in the course of *L .major* infection. CD8$^+$ cDCs did not show any difference between the two strains (A & C). CD11b$^+$ CD8$^-$ cDCs population were significantly smaller in B6.TNF$^{-/-}$ mice during the course of *L. major* infection (A & D)., with decreased expression of MHCII and CD11c expression compared to B6.WT mice. Five B6.WT and B6.TNF$^{-/-}$ mice were used each time point. Error bars represent the mean ± SEM from three independent experiments. The *p* values were calculated using a two tailed Mann-Whitney U test (**p<0.01).

5.3 Conclusion and discussion

Although previous research found *L. major* induced splenic leishmaniasis in the absence of TNF, it did not explain why the loss of TNF leads to splenic infection [132]. This chapter has provided several insights into the reasons, especially the dysregulation of spleen cells responsible for *L. major*-induced visceral spleen infection in the absence of TNF.

For this study, choosing a proper control for *L. major*-infected spleen from B6.TNF$^{-/-}$ mice was challenging because *L. major* infection does not induce visceral leishmaniasis in B6.WT mice. Therefore, we could not directly compare cell population changes between B6.WT and B6.TNF$^{-/-}$ mice over the course of the disease. One possibility would be to using *L. donovani*-induced visceral infection model, however this well-described model is different from *L. major* induced leishmaniasis. Therefore, we chose BALB/c strain as a positive control for B6.TNF$^{-/-}$ mice during *L. major*-induced infection, as it is a susceptible strain for visceral *L. major* infection. Although they have TNF gene, they fail to control disease progression developing visceral organ infection due to its Th2-dominated immune response. Therefore, this overcame the difficulties that *L. donovani* model brought and limitation when only having B6.WT mice. This control group allowed us to appropriately evaluate how the visceral infection in B6.TNF$^{-/-}$ mice is close to normal visceral infection and offered a way to the insight into the requirement for TNF in parasite control as well as the generation of various cell population to compare with B6.WT strain.

The enlarged spleen is a typical hallmark for visceral leishmaniasis [91]. In line with that, we found spleen at day 42 from B6.TNF$^{-/-}$ mice was extremely large, around 2-3 times bigger compared to B6.WT mice, and the weight was significant higher, suggesting spleen infection

occurred in B6.TNF$^{-/-}$ mice upon *L. major* challenge. In order to confirm that parasites exist in the spleen, infection load was analyzed by limiting dilution assay. *L. major* were found in the spleens from B6.TNF$^{-/-}$ mice, reaching around 150000 per gram of tissue at the late stage of infection, while parasites in B6.WT mice were rarely observed. This information demonstrated loss of TNF lead to splenic leishmaniasis, but also provided the valuable addition that an overload of parasites may destroy the structure of spleen and contribute to its dysfunction. It has been reported a higher load of parasites lead to the disruption of splenic architecture [236]. Therefore, one aim was to determine whether the spleen had changes in structure in the absence of TNF during *L. major* infection.

The spleen acts primarily as a filter to purify the blood by removing damaged cells, cellular debris and some blood-borne pathogens [216]. Intact spleen structure is very important and tightly associated with its function. TNF is a major cytokine responsible for maintaining normal spleen structure. Loss of TNF abolished the capacity of B cells to form normal folliclar structure and germinal centre formation [42, 237]. Therefore, it was not surprising that the microarchitecture was poorly demarcated in B6.TNF$^{-/-}$ mice after infection. The white pulps and red pulps were rarely detected, and a large number of immune cells were dispersed throughout the spleen tissue. Although spleen from BALB/c mice displayed the progressive development of splenic pathology including the disruption of tissue anatomy and reduced numbers of germinal centre, their basic structure was still detectable compared to B6.TNF$^{-/-}$ mice, which confirmed the importance of TNF in retaining and regulating the fine architectural organization of splenic lymphoid tissue upon infection.

A normal spleen has a well-organized structure of red pulp and white pulp, partitions consisting of a range of immune cells that protect against infection. Despite our preliminary examination of the change to spleen architecture in this chapter, it is imperative that future work examines any change of immune cells in the spleen of B6.TNF$^{-/-}$ during infection.

Cell mediated immunity, specifically CD4^{+} and CD8^{+} T cell, has been reported to participate in killing of intracellular parasites. *Leishmania* specific CD4^{+} T cells release IFN-γ, which limits parasite replication in visceral leishmaniasis [238], and immune-depletion of CD4^{+} T cells completely reversed the protection and failed to restrain parasite replication [239]. CD8^{+} T cells are able to directly lyse infected cells and can produce high levels of IFN-γ, but they also contribute to tissue repair, thereby facilitating parasite replication [240-242]. An increased CD8^{+} cell population and a lower ratio of CD4/CD8 T cells were described in visceral leishmaniasis [243, 244]. Our result has not shown any change of CD4^{+} or CD8^{+} T cell percentage, and their ratio was similar during the whole infection process in B6.WT and B6.TNF$^{-/-}$ mice. Similarly, the CD4^{+}/ CD8^{+} T cell ratio from BALB/c mice also did not show any difference after infection. These data were consistent with previous research showing the absence of TNF did not change the ratio of B to T cells in the spleen [42, 148], and also indicate the dysfunction of the spleen may not be relevant to T cell and B cell immunity.

Of note, Medina-Colorado *et al* claimed that splenic CD4^{+} T cells may have a mixed effector-regulatory phenotype during progressive visceral leishmaniasis, which impairs macrophage effector function through inhibitory receptor expression [245]. Although we showed TNF did not affect the ratio of CD4^{+} and CD8^{+} cells, we did not demonstrate if TNF led to a mixed CD4^{+} T cells phenotype that potentially could promote disease progression.

This being the case, splenic adaptive immune cells have not been found impaired in the absence of TNF during *L. major*-induced leishmaniasis, it would be some other cell population changes are leading to the disease progression. The most likely target cell we hypothesis is innate immune cells especially APC cells, because any interruption of the antigen presenting process or interaction process between APC and T cell could also lead to failure to control parasites. Detection by flow cytometry clearly showed the absence of TNF affected monocyte/macrophage population. Macrophages are critical in the regulation of anti-leishmanial immunity, but the dynamics of macrophages in the spleen during leishmaniasis are still poorly understood. It was reported that *Leishmania* amastigotes were rapidly taken up by macrophages in the spleen of BALB/c mice [34], and around 50% of parasites are killed by macrophages in the marginal zone in the first 24 hours after infection of the spleen [217]. These cells may correspond to the cell found in F4/80^{-} marginal zone, which displayed DC-phenotype and scavenger the receptor MARCO and are proficient in the capture and killing of the pathogen [246]. We have shown here that splenic macrophages, that determines the outcome of the immune response against cutaneous leishmaniasis, were affected in the absence of TNF. Although CD11b^{+} cells were elevated in B6.WT mice, the number of F4/80^{hi} of CD11b^{+} cells were much higher in B6.TNF$^{-/-}$ mice with the low expression of CCR2 and Ly6C, indicating this population was resident macrophage of spleen. This result was contrary to previous reports that splenic macrophages were efficient in killing the parasites, but it may be explained by these macrophages acquiring M2-like phenotype which failed to eliminate the parasites in the absence of TNF. Of note, Martínez-Pomares *et al* demonstrated that splenic macrophage from red pulp expressed high levels of CD206 [247], and further Swirski *et al*. claimed undifferentiated monocytes residing in the spleen can express mannose receptor, that not only

mature macrophages [54]. Previously, we have demonstrated loss of TNF skewed the monocyte differentiation into M2 macrophages, and this finding to some extent supported what we found in the present study, that an elevated splenic macrophage population may exhibit M2 like phenotype. Further work is needed to understand the heterogeneity of this macrophage population in spleen.

In addition to monocyte/macrophage, DCs are an important population for defending against *Leishmania* infection. They arise from common dendritic progenitors (CDP) and are capable of sensing the pathogen, producing a number of cytokines and presenting antigen to T cells and determine the immune response [248]. There are two main functional groups of DCs, pDCs and cDCs. During inflammation, another subset of DC (named as monocyte-derived DCs or inflammatory DC) are generated, which has a different origin from pDCs and cDCs. pDC is the main source of IL-12, the key cytokine in controlling the parasites in the early stage of infection and is required for IFN-γ production in visceral leishmaniasis [249]. A single i.v injection of $5 \times 10^4 - 5 \times 10^5$ pDCs as vaccine has been showed sufficient to induce protective immunity against single or repeated *L. major* parasite challenges in susceptible BALB/c mice [250]. Depletion of pDCs demonstrated that pDCs are required for the development of an adequate innate immune response, including recruitment of polymorphonuclear leukocytes, monocytes, and macrophages to the site of *Leishmania* infection [251]. In line with these findings, our results here found the decreased percentage of pDCs in the absence of TNF, indicating the aberrant pDCs may be one reason affecting the immune response against *Leishmania*. It is still controversial how TNF affects maturation and differentiation of immature pDCs [252]. Palucka *et al* showed a cross-regulation exists between TNF and IFN-α, the latter is produced principally by pDC. They found TNF inhibited not only the generation

of pDCs from $CD34^+$ hematopoietic progenitors but also IFN-α release by immature pDCs exposed to influenza virus [253]. In contrast, Angelot *et al* showed the exposure of pDC to TNF-induced endothelial micro-particles increased the secretion of IFN-α by pDC and promoted pDC maturation, subsequently favouring Th1 polarization, showing the co-operative relationship between TNF and IFN-α [254]. In our *L. major* infection model, pDCs are positively regulated by TNF, indicating the interaction between TNF and pDCs may vary under different inflammatory conditions, different organs, and different species.

In addition, we found a cell population characterized by $B220^{hi}CD11b^{med}CD11c^{hi}Ly6C^-$, which has not been defined in any previous publication. The number of these novel cells was significantly less in $B6.TNF^{-/-}$ mice compared to B6.WT mice. How best to define cell populations with limited markers is always difficult. This population was proposed to consist of IFN-γ-producing killer dendritic cells, which have previously been investigated in relation to tumor immunity [255, 256]. However, since we depleted $NK1.1^+$ cells and confirmed the purity of the negative cell population beforehand, it is unlikely they express NK cell markers. In terms of the increased $CD11b^+$ monocytes in B6.WT mice, we were considering whether there was any correlation between $CD11b^+$ monocytes and this novel cell population. Ly6C expression of monocytes is lost during differentiation into DCs, which in consistent with our finding here. Furthermore, it was reported that a $CD11c^+B220^+Gr\text{-}1^+$ cells in mouse spleen displayed characteristics of pDCs, similarly to what we described [257]. Therefore, this novel cell population may be a monocyte-derived DC displaying a pDC phenotype. Although $Gr1^+$ (including $Ly6G^+$ and $Ly6C^+$) expression was found, which may be due to other cells contamination in their model [257]. pDCs normally produce type I interferons that participate in anti-pathogen responses [258]. As we did not examine the cytokine secretion and their

capacity of antigen presenting of this population, we were unable to confirm and accurately define this novel population. Indeed, inflammation triggers recruitment and differentiation of monocytes into microbicidal macrophages and DCs [62], and TNF is also required for the recruitment and accumulation of DCs with neutralization of TNF reducing myeloid DC [259]. Mice without the TNF gene fail to orchestrate the formation of DCs and initiate the Th1 immune response at the site of *L. major* infection [160]. If this holds true, that can explain lower $B220^{hi}CD11b^{med}CD11c^{hi}Ly6C^{-}$ number in $B6.TNF^{-/-}$ mice, and which may be another important reason that B6.TNF mice fail to control the parasites in the spleen and eventually succumb to the infection.

Chapter 6.

Final Discussion and Conclusion

6.1 Final discussion

Leishmaniasis is a parasitic disease caused by the heterogeneous genus of *Leishmania* spp. According to the data from WHO, more than 12 million people worldwide are infected with *Leishmania*, and 1.3 million new cases are reported annually [1]. Clinically, three basic forms of leishmaniasis are recognized: cutaneous, mucocutaneous and visceral leishmaniasis.

As TNF has a key role in mediating host protection against leishmaniasis, the use of anti-TNF agents may result in increased susceptibility new infection or reactivation of a latent infection of leishmaniasis. It was reported that cutaneous [130, 260] or visceral leishmaniasis [125-127] occurred in RA Patients treated with infliximab. Similarly, AS patients also have been found leishmaniasis involving usage of anti-TNF regents [261]. Interestingly, mucocutaneous leishmaniasis was reported in an AS patient on adalimumab, that was induced by *L. infantum*, a species which normally leads to visceral leishmaniasis [129], indicating that the type of manifestation and degree of dissemination of the parasites depend on the virulence of the *Leishmania* species involved as well as the immune response of the host. Therefore, as a matter of fact, patients should be adequately treated with anti-TNF agents and monitored no matter of the early signs of infection.

Although these case reports indicated that TNF inhibitor is associated with an increased risk of *Leishmania* infection, there is scarce literature on why the loss of TNF leads to leishmaniasis mainly due to the absence of control. Therefore, researchers established experimental animal models, including the mouse infection model, in order to provide more helpful data regarding the host response and pathogenesis that parallels human disease and to characterize the underlying mechanisms.

Experimental infection of mice with *L. major* promastigotes has allowed understanding of the immunologic mechanisms governing resistance of the C57BL/6 strain and susceptibility of the BALB/c strain to the infection. Susceptibility is tightly correlated with a Th2 type of immune response, while the immunity in resistant mice depends on the development of a Th1 biased immune response. However, after deletion of the TNF gene, normally resistant B6.WT mice are unable to control a cutaneous infection with *L. major*, which essentially reproduces the pathology observed in patients receiving anti-TNF therapy. Therefore, based on this gene knock-out mouse model, this project was designed to investigate how the loss of TNF affect the immune response during *L. major*-induced cutaneous leishmaniasis.

Internal organ infection normally occurs in *L. donovani*-induced visceral leishmaniasis. However, infected liver and spleen were found in B6.TNF$^{-/-}$ mice in *L. major*-induced cutaneous leishmaniasis, indicating that the immune response was impaired in the absence of TNF that results in failure to kill the parasites and control the parasite spreads. The resolution of parasite infection in B6.WT mice is mainly dependent on Th-1 mediated immune responses for killing of *L. major* within macrophages. The most efficient mechanism of parasite killing involves the production of IFN-γ by CD4^{+} Th1 cells, which contributes to macrophage activation and NO production [9, 158, 159, 262]. In the present study, although IFN-γ production was not affected by the loss of TNF as published earlier [133], it did not prevent the progression of the disease. Recently, a paradoxical expression of IFN-γ was found in the spleen during visceral leishmaniasis. It has shown that IFN-γ-induced transcripts were upregulated in the spleen, but not in the splenic macrophages. Moreover, IFN-γ was found promote *Leishmania* growth and upregulate counter-regulator molecule such as Arg-1 in

splenic macrophage, which made the host more susceptible [263]. As such, it is likely to support our finding that higher IFN-γ was insufficient in mediating a protective immune response in macrophage and halting the disease progression.

The demonstration that high levels of expression of IFN-γ and intact Th-1 immunity in B6.TNF$^{-/-}$ mice did not explain why the mice were still susceptible to *L. major*. Whether the innate immune response is affected by the loss of TNF needs to be examined. Macrophages act as the reservoir for parasites replication but also are the most efficient effector cells for parasite killing. In the liver, hepatic macrophages consist of Kupffer cells and additional recruited monocyte-derived macrophages involved in pathogen defense. Kupffer cells are derived from embryonic cells and self-maintain independently from hematopoietic input under steady state [51]. They serve as sentinels to sense insults and initiate inflammation. It was demonstrated that Kupffer cells underwent necroptosis in early infection with *Listeria*, triggering recruitment of monocytes and inflammation [163]. Unsurprisingly, in the slower moving *L. major* infection we did not see a significant decline in the numbers of Kupffer cells nor did we detect a significant genotype-dependent changes of Kupffer cells between B6.WT and B6.TNF$^{-/-}$ mice during the course of infection (with the exception of day 21 p.i). More Kupffer cells were found in B6.WT mice at day 21 p.i., which can be explained by the loss of TNF impaired the onset of the immune response during cutaneous leishmaniasis [133].

The work in chapter 3 provided evidence that Mo-M might be the key reason for visceral liver infection in the absence of TNF. Mo-M plays a protective role or contribute to disease progression depending on different circumstances affecting their polarization. In the bacteria infection, Mo-M displayed M2 phenotype upon IL-4 signaling, replaced necrotized Kupffer

cells and sustained the homeostasis [163], indicating that Mo-M also can be polarized into M1 or M2 phenotype in response to different signals or microbes. In leishmaniasis, a monocytic population was identified as Mo-M in the skin and draining lymph nodes in B6.TNF$^{-/-}$ mice and exhibited a phenotype that was CD11b^{+}Ly-6C^{lo}CCR2^{lo}iNOS^{lo} and harbored a large number of parasites, indicating that Mo-M might contribute to parasite replication during leishmaniasis [95]. In the liver, which constitutes a major target organ of a visceralized *L. major* infection in immune-incompetent mice, Mo-M has not yet been addressed in detail. Our data firstly defined liver Mo-M and demonstrated its accumulation associated with mouse susceptibility. Monocyte recruitment was discontinued at the late stage of infection, indicating the insult has been cleared and no further requirement of monocyte influx in B6.WT mice. However, a continual accumulation of monocytes, as well as Mo-M, was found in the B6.TNF$^{-/-}$ mice, which further showed that the severe liver infection occurs through the disease progression. Although there was consistent recruitment of immune cells, the disease progression was not stopped. M2 macrophage has been demonstrated to impede protective immunity to leishmaniasis, and impairment of M2 macrophage dramatically delays disease progression [264]. With further detailed investigation, this unique Mo-M was found to exhibit an M2 phenotype with highly expressed CD206, Arg-1 as well as IL-6. Also, observations from B6.TNF$^{-/-}$ mice showed parasites inside and on the surface of Mo-M that further supported M2 phenotype of Mo-M. This investigation conclusively demonstrated the concept that the M2-suppressing role of TNF is not organ- or tissue-specific and, together with the observation of M2-like cells in the spleen of *L. monocytogenes* infected TNF-deficient mice supports the notion of a fundamental, yet so far undescribed biological activity of this cytokine [134, 182, 265]

Expression of iNOS is the primary anti-*Leishmania* mechanism in mice, and it has been shown that higher levels of iNOS in macrophage contributes to the resolution of Leishmania [266-269]. Arg-1 competes for the same substrate arginine of iNOS by converting the amino acid into ornithine and urea, contributing the growth of *Leishmania* parasites. Therefore, the balance between iNOS and Arg-1 directly determines the outcome of leishmaniasis. In the unique M2-like Mo-M, we found mRNA level of iNOS was competitively inhibited and Arg-1 was super highly expressed. In the liver tissue, iNOS expression was also lower in B6.TNF$^{-/-}$ mice within more *L. major* parasites dispersed, which indicated that absence of TNF led the mice unable to produce the leishmanicidal effector molecule NO and consequently caused the progressive visceralization of the parasites. This finding contradicted the previous findings that showed that TNF had little effect on the expression of iNOS. An *L. major* infection of a TNFR1-negative mouse strain showed that in the absence of the pro-inflammatory signaling pathway, these mice developed persistent lesions but controlled the pathogen with sustained iNOS [131, 270]. Furthermore, the monocytic population of lymph nodes in B6.TNF$^{-/-}$ mice had a progressive and ultimately fatal infection [132], despite a strong Th1 response which was characterized by a hyper-expression of IFN-γ and the presence of iNOS [95, 133]. The differences in the published outcomes of these infection experiments were likely due to variations of the genetic background of the parasite strains [271]. The apparent contradiction of the presence of iNOS in TNF-deficient mice and their concurrent susceptibility to *L. major* infection was explained with the observation that TNF caused a direct suppression of Arg-1 expression and of other molecules associated with an alternative activation of myeloid cells by TNF. In the absence of TNF, this permitted a co-expression of Arg-1 and iNOS. Since both enzymes share L-arginine as a substrate, co-expression in macrophages would cause a

competition for the substrate. Consequently, loss of TNF reduced the production of NO significantly resulting in fatal leishmaniasis [134].

Another key finding from these investigation is increased IL-6 mediated monocyte/macrophage differentiation. It has been shown that IL-6 not only acts as a classical pro-inflammatory cytokine but also involves in the development of M2 macrophages. IL-6 is highly expressed with increasing levels of TNF in the patients with autoimmune disease [272-277], but endogenous IL-6 was also reported to control the inflammation in the local and systemic acute inflammatory response [143]. IL-6-mediated alternative macrophage activation has been demonstrated to inhibit obesity-associated insulin resistance and endotoxemia, thus maintaining homeostasis [146]. Therefore, the function of IL-6 may vary depending on the diseases and the cytokine environments. During human leishmaniasis, higher levels of IL-6 was observed in visceral *Leishmania* infection, and its increased expression tightly associated with disease severity [193, 197]. However, other evidence raised the possibility that IL-6 may be not even required or contribute to restraining *Leishmania* infection [179, 180, 199]. In the present *in vitro* study, IL-6 skewed monocyte differentiation from DCs to macrophages, and further facilitated M2 macrophage polarization, which in line with previous literature [191, 192, 203]. This can refer to the results found IL-6 increased in Mo-M with high level of CD206 and Arg-1 *in vivo*. IL-6-mediated Arg-1 activation as well as macrophages and DCs dysfunction have been reported as the critical mechanisms for inducing dysfunction of the immune system in the tumor [278], and inhibition of IL-6 contributed to NO production [279]. Thus, the evidence may explain the correlation of higher level of IL-6 and Arg-1 in Mo-M and increased susceptibility to *L. major* infection, and indicate IL-6 is an anti-inflammatory cytokine to contribute to the parasite growth in B6.TNF$^{-/-}$ mice.

The question still remains is why is IL-6 elevated in B6.TNF$^{-/-}$ mice? Taking into account that IL-6 has an anti-inflammation role and that the regulatory balance between IL-6 and TNF has been previously reported in bacterial infection [202], it is likely that absence of TNF led to increased of IL-6. *In vitro*, we found TNF reversed IL-6-mediated monocyte differentiation from macrophages to DCs, and inhibited IL-6-facilitated M2 macrophage polarization through increasing CSFR internalization, demonstrating a counter balance function between TNF and IL-6.

IL-6 exerts numerous biological processes by formation a complex with its two receptors: type I transmembrane glycoprotein IL-6R and type I transmembrane signal transducer protein gp130. When IL-6 first bind to the membrane-bound non-signaling receptor IL-6R (mIL-6R), homodimerization of gp130 is induced and the dimerization of IL-6/mIL-6R/gp130 is subsequently formed, which then activates Janus activated kinase (JAK) and induce JAK auto-phosphorylates, that subsequently mediates signal transducer and activator of transcription (JAK/STAT), ERK and PI3K signaling pathways [139]. However, mIL-6Rα is expressed exclusively on the surface of hepatocytes and some myeloid and lymphoid cell populations such as monocytes and T cells [140].Thus, most pro-inflammatory functions of IL-6 are mediated through a second IL-6-dependent signaling pathway via the soluble IL-6R (sIL-6R) in the IL-6 trans-signaling pathway which can trigger IL-6-mediated response in cells negative for mIL-6R [280]. Gp130-STAT3 signaling is very important in IL-6 mediated cell differentiation including monocyte and macrophage [203, 212]. In the present study, upon IL-6 stimulation, mIL-6R, gp130 and STAT3 were all activated, and in turn, they were inhibited

when treated with additional TNF, thus illustrating the regulatory role of IL-6 in mediating monocyte/macrophage differentiation through mIL-6R/gp130 and STAT3.

M2 macrophage polarization was classically driven by IL-4-induced STAT6 activation [281], and STAT6 phosphorylation was critically important for Arg-1 expression during visceral leishmaniasis [282, 283]. In our study, we also found IL-4-STAT6 activation in response to IL-6 stimulation, while the additional presence of TNF caused a significant reduction of STAT6 phosphorylation. This result is consistent with the STAT3 regulation, indicating STAT3 and STAT6 may co-ordinately regulate each other in contributing to M2 macrophage polarization [146, 215]. Of note, it has also reported that the parasite-promoting effect of IFN-γ is mediated through STAT3 activation [263, 284, 285].Therefore, it is possible that higher IFN-γ contribute to M2 macrophage polarization via STAT3, eventually leading to leishmaniasis. This multiple-interaction among different cytokines and signaling pathways in M2 macrophage polarization needs to be addressed in the future experiments, which may allow better understanding of M2 macrophage development in complex environments, and potentially offer a rational target for reducing M2-mediated host pathogenesis.

Liver and spleen are the two main target organs during visceral leishmaniasis. In the spleen, the immune system fails to clear parasites and instead, a lifelong chronic infection persists associated with immunopathology [286]. In our study, splenomegaly is typical effect of infection symptom in B6.TNF$^{-/-}$ mice during cutaneous leishmaniasis. A higher load of parasites led to the disruption of the spleen architecture [236], which can directly impair normal spleen function. TNF is one of the cytokines contributing to maintenance of normal tissue structure, including spleen. In the absence of TNF, the microarchitecture of the splenic white

pulp is abnormal, and splenic germinal centres failed to develop upon stimulation [237]. Therefore, it was not surprising that splenomegaly occurred in the absence of TNF during cutaneous leishmaniasis.

Compared to the liver, the investigation of immune response in the spleen is less developed due to complex heterogeneity of different splenic cell population. Macrophages are critical in the regulation of anti-leishmanial immunity, the dynamics of splenic macrophages during leishmaniasis are poorly understood. In the present study, we found the proportion of splenic macrophages were comparatively greater in the absence of TNF, with higher expression of F4/80 and low expression of CCR2 and Ly6C indicating their resident macrophage phenotype. This result was contrary to previous reports that splenic macrophages were efficient to at killing the parasites [217, 246], but it may be explained that these macrophages acquired as M2-like phenotype and failed to eliminate the parasite in the absence of TNF. Considering the different resident macrophage population such as marginal zone macrophages and metallophilic macrophages, further work to distinguish the various populations by specific cell markers is required, and would allow better understanding regarding which macrophages would be affected by loss of TNF, and potentially help to control the infection.

Dendritic cells comprise several subsets, with different DC types having distinct roles in initiating immunity to specific pathogens. Plasmacytoid DCs are able to induce Th-1 responses, and vaccination of susceptible mice with pDCs induced highly effective T cell-mediated immunity against *L. major* infection [250]. This could explain the higher pDCs population found in B6.WT mice, and also indicate the absence of TNF led to decrease the number of pDCs. Interestingly, compared to B6.WT mice, a significant decreased cell population was found in B6.TNF$^{-/-}$ mice, which expressed B220^{hi}CD11b^{med}CD11c^{hi}Ly6C and has not been

defined previously. This population was proposed to be derived from monocytes based on the higher levels of $CD11b^+$ expression. Although Ly6C expression is negative for this population, it was reported that this marker is lost during differentiation into DCs [287]. Furthermore, a $CD11c^+B220^+Gr\text{-}1^+$ cells in mouse spleen which displayed characteristics of pDCs was reported, which is quite similar to the cells described in our study [257]. With the current data, we cannot definitely state that this novel population are monocyte-derived DCs which have a pDC phenotype, but further research need to be undertaken to identify their characteristics using more specific cell markers and their function are assessed by isolation and vaccination of $B6.TNF^{-/-}$ mice. Although investigation of DCs in the spleen raised more questions than answers, these preliminary results may offer a good starting point to demonstrate a TNF impaired DCs population and provide some clues for the further investigation.

6.2 Conclusion

The absence of TNF causes lethal infection by *L. major* in resistant mice B6.WT mice, but the underlying mechanism has so far remained elusive. In this thesis, I have focused on examining visceral organ infection during cutaneous leishmaniasis. I hypothesized that loss of TNF caused impaired monocyte/macrophage differentiation in the liver and spleen and consequently had a fatal outcome after liver and spleen involvement. As a result of this investigation, this thesis has presented evidence for the critical role of TNF in pathogen defence by the innate system. I have characterised the role of TNF in alternative macrophage activation, the counter-regulation between TNF and IL-6, and the underlying mechanism of their interaction in *L. major*-induced leishmaniasis.

This is the first study to investigate internal organ infection during cutaneous leishmaniasis and to examine how TNF does orchestrate cell differentiation and the protective immune response

against the pathogen. In doing so, this study has extended our previous knowledge of the loss of TNF leading to fatal outcomes during cutaneous leishmaniasis and provided insight into innate cell dysregulation and abnormal differentiation that occurs during leishmaniasis in the absence of TNF. As a result of this work, I propose that loss of TNF directly causes visceral organ infection, due to dysregulation and impaired differentiation of innate cells. This thesis also potentially explains the increased risk of opportunistic infection and relapses in patients receiving anti-TNF treatment, and will potentially help to maximize therapeutic benefits while minimizing adverse effects from anti-TNF treatments.

Chapter 7.

References

Uncategorized References

1. WHO, *Leishmaniasis home.* 2014.
2. Ribeiro-Gomes, F.L. and D. Sacks, *The influence of early neutrophil-Leishmania interactions on the host immune response to infection.* Front Cell Infect Microbiol, 2012. **2**: p. 59.
3. Baldwin, T., et al., *Wound healing response is a major contributor to the severity of cutaneous leishmaniasis in the ear model of infection.* Parasite Immunol, 2007. **29**(10): p. 501-13.
4. Aderem, A. and D.M. Underhill, *Mechanisms of phagocytosis in macrophages.* Annu Rev Immunol, 1999. **17**: p. 593-623.
5. Sehgal, G., et al., *Lectin-like inhibition of immune complex receptor-mediated stimulation of neutrophils. Effects on cytosolic calcium release and superoxide production.* J Immunol, 1993. **150**(10): p. 4571-80.
6. Belkaid, Y., et al., *A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity.* J Immunol, 2000. **165**(2): p. 969-77.
7. Green, S.J., et al., *Leishmania major amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha.* J Immunol, 1990. **145**(12): p. 4290-7.
8. Wei, X.Q., et al., *Altered immune responses in mice lacking inducible nitric oxide synthase.* Nature, 1995. **375**(6530): p. 408-11.
9. Liew, F.Y., Y. Li, and S. Millott, *Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of Leishmania major through the induction of nitric oxide.* J Immunol, 1990. **145**(12): p. 4306-10.
10. Gordon, S., *Alternative activation of macrophages.* Nat Rev Immunol, 2003. **3**(1): p. 23-35.
11. Iniesta, V., et al., *Arginase I induction during Leishmania major infection mediates the development of disease.* Infect Immun, 2005. **73**(9): p. 6085-90.
12. Tasew, G., et al., *Systemic FasL and TRAIL neutralisation reduce leishmaniasis induced skin ulceration.* PLoS Negl Trop Dis, 2010. **4**(10): p. e844.
13. van Zandbergen, G., et al., *Cutting edge: neutrophil granulocyte serves as a vector for Leishmania entry into macrophages.* Journal of immunology, 2004. **173**(11): p. 6521-5.
14. Kane, M.M. and D.M. Mosser, *Leishmania parasites and their ploys to disrupt macrophage activation.* Curr Opin Hematol, 2000. **7**(1): p. 26-31.
15. Charmoy, M., et al., *Leishmania major induces distinct neutrophil phenotypes in mice that are resistant or susceptible to infection.* J Leukoc Biol, 2007. **82**(2): p. 288-99.
16. Blos, M., et al., *Organ-specific and stage-dependent control of Leishmania major infection by inducible nitric oxide synthase and phagocyte NADPH oxidase.* Eur J Immunol, 2003. **33**(5): p. 1224-34.
17. Guimaraes-Costa, A.B., et al., *Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps.* Proc Natl Acad Sci U S A, 2009. **106**(16): p. 6748-53.

18. Rodrigues, V., et al., *Regulation of immunity during visceral Leishmania infection.* Parasit Vectors, 2016. **9**: p. 118.
19. Engwerda, C.R., M. Ato, and P.M. Kaye, *Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis.* Trends Parasitol, 2004. **20**(11): p. 524-30.
20. Nieto, A., et al., *Mechanisms of resistance and susceptibility to experimental visceral leishmaniosis: BALB/c mouse versus Syrian hamster model.* Vet Res, 2011. **42**: p. 39.
21. McElrath, M.J., H.W. Murray, and Z.A. Cohn, *The dynamics of granuloma formation in experimental visceral leishmaniasis.* J Exp Med, 1988. **167**(6): p. 1927-37.
22. Murray, H.W., *Tissue granuloma structure-function in experimental visceral leishmaniasis.* Int J Exp Pathol, 2001. **82**(5): p. 249-67.
23. McFarlane, E., et al., *Neutrophils contribute to development of a protective immune response during onset of infection with Leishmania donovani.* Infect Immun, 2008. **76**(2): p. 532-41.
24. Faleiro, R.J., et al., *Immune regulation during chronic visceral leishmaniasis.* PLoS Negl Trop Dis, 2014. **8**(7): p. e2914.
25. Flynn, J.L., et al., *Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice.* Immunity, 1995. **2**(6): p. 561-72.
26. Bean, A.G., et al., *Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin.* J Immunol, 1999. **162**(6): p. 3504-11.
27. Mohan, V.P., et al., *Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology.* Infect Immun, 2001. **69**(3): p. 1847-55.
28. Lin, P.L., et al., *Tumor necrosis factor and tuberculosis.* J Investig Dermatol Symp Proc, 2007. **12**(1): p. 22-5.
29. Saunders, B.M. and A.M. Cooper, *Restraining mycobacteria: role of granulomas in mycobacterial infections.* Immunol Cell Biol, 2000. **78**(4): p. 334-41.
30. Murray, H.W., et al., *Visceral leishmaniasis in mice devoid of tumor necrosis factor and response to treatment.* Infect Immun, 2000. **68**(11): p. 6289-93.
31. Tumang, M.C., et al., *Role and effect of TNF-alpha in experimental visceral leishmaniasis.* J Immunol, 1994. **153**(2): p. 768-75.
32. Murray, H.W., et al., *Visceral Leishmania donovani infection in interleukin-13-/- mice.* Infect Immun, 2006. **74**(4): p. 2487-90.
33. Murray, H.W., et al., *Experimental visceral leishmaniasis: production of interleukin 2 and interferon-gamma, tissue immune reaction, and response to treatment with interleukin 2 and interferon-gamma.* J Immunol, 1987. **138**(7): p. 2290-7.
34. Gorak, P.M., C.R. Engwerda, and P.M. Kaye, *Dendritic cells, but not macrophages, produce IL-12 immediately following Leishmania donovani infection.* Eur J Immunol, 1998. **28**(2): p. 687-95.
35. Stanley, A.C. and C.R. Engwerda, *Balancing immunity and pathology in visceral leishmaniasis.* Immunol Cell Biol, 2007. **85**(2): p. 138-47.

36. Ato, M., et al., *Loss of dendritic cell migration and impaired resistance to Leishmania donovani infection in mice deficient in CCL19 and CCL21.* J Immunol, 2006. **176**(9): p. 5486-93.
37. Zhu, J., H. Yamane, and W.E. Paul, *Differentiation of effector CD4 T cell populations (*).* Annu Rev Immunol, 2010. **28**: p. 445-89.
38. Kaech, S.M. and W. Cui, *Transcriptional control of effector and memory CD8+ T cell differentiation.* Nat Rev Immunol, 2012. **12**(11): p. 749-61.
39. Smith, L.E., M. Rodrigues, and D.G. Russell, *The interaction between CD8+ cytotoxic T cells and Leishmania-infected macrophages.* J Exp Med, 1991. **174**(3): p. 499-505.
40. Smelt, S.C., et al., *Destruction of follicular dendritic cells during chronic visceral leishmaniasis.* J Immunol, 1997. **158**(8): p. 3813-21.
41. Engwerda, C.R., et al., *A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during Leishmania donovani infection.* Am J Pathol, 2002. **161**(2): p. 429-37.
42. Korner, H., et al., *Tumor necrosis factor sustains the generalized lymphoproliferative disorder (gld) phenotype.* J Exp Med, 2000. **191**(1): p. 89-96.
43. Ato, M., et al., *Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis.* Nat Immunol, 2002. **3**(12): p. 1185-91.
44. Nolte, M.A., et al., *A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp.* J Exp Med, 2003. **198**(3): p. 505-12.
45. Cyster, J.G., *Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs.* J Exp Med, 1999. **189**(3): p. 447-50.
46. Murphy, M.L., et al., *IL-10 mediates susceptibility to Leishmania donovani infection.* Eur J Immunol, 2001. **31**(10): p. 2848-56.
47. Murray, H.W., et al., *Determinants of response to interleukin-10 receptor blockade immunotherapy in experimental visceral leishmaniasis.* J Infect Dis, 2003. **188**(3): p. 458-64.
48. De Kleer, I., et al., *Ontogeny of myeloid cells.* Front Immunol, 2014. **5**: p. 423.
49. Guilliams, M., et al., *Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny.* Nat Rev Immunol, 2014. **14**(8): p. 571-8.
50. Steinman, R.M. and M.D. Witmer, *Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice.* Proc Natl Acad Sci U S A, 1978. **75**(10): p. 5132-6.
51. Yona, S., et al., *Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.* Immunity, 2013. **38**(1): p. 79-91.
52. Ginhoux, F. and S. Jung, *Monocytes and macrophages: developmental pathways and tissue homeostasis.* Nat Rev Immunol, 2014. **14**(6): p. 392-404.
53. Auffray, C., M.H. Sieweke, and F. Geissmann, *Blood monocytes: development, heterogeneity, and relationship with dendritic cells.* Annu Rev Immunol, 2009. **27**: p. 669-92.
54. Swirski, F.K., et al., *Identification of splenic reservoir monocytes and their deployment to inflammatory sites.* Science, 2009. **325**(5940): p. 612-6.
55. Cecchini, M.G., et al., *Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse.* Development, 1994. **120**(6): p. 1357-72.

56. Dai, X.M., et al., *Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects.* Blood, 2002. **99**(1): p. 111-20.
57. Serbina, N.V., et al., *Monocyte-mediated defense against microbial pathogens.* Annu Rev Immunol, 2008. **26**: p. 421-52.
58. Yang, J., et al., *Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases.* Biomark Res, 2014. **2**(1): p. 1.
59. Varol, C., A. Mildner, and S. Jung, *Macrophages: development and tissue specialization.* Annu Rev Immunol, 2015. **33**: p. 643-75.
60. Auffray, C., et al., *Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior.* Science, 2007. **317**(5838): p. 666-70.
61. Peng, Y., Y. Latchman, and K.B. Elkon, *Ly6C(low) monocytes differentiate into dendritic cells and cross-tolerize T cells through PDL-1.* J Immunol, 2009. **182**(5): p. 2777-85.
62. Serbina, N.V., et al., *TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection.* Immunity, 2003. **19**(1): p. 59-70.
63. Nahrendorf, M., et al., *The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions.* J Exp Med, 2007. **204**(12): p. 3037-47.
64. Sunderkotter, C., et al., *Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response.* J Immunol, 2004. **172**(7): p. 4410-7.
65. Varol, C., et al., *Monocytes give rise to mucosal, but not splenic, conventional dendritic cells.* J Exp Med, 2007. **204**(1): p. 171-80.
66. Dal-Secco, D., et al., *A dynamic spectrum of monocytes arising from the in situ reprogramming of CCR2+ monocytes at a site of sterile injury.* J Exp Med, 2015. **212**(4): p. 447-56.
67. Arnold, L., et al., *Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis.* J Exp Med, 2007. **204**(5): p. 1057-69.
68. Altin, J.G. and E.K. Sloan, *The role of CD45 and CD45-associated molecules in T cell activation.* Immunol Cell Biol, 1997. **75**(5): p. 430-45.
69. van Lochem, E.G., et al., *Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts.* Cytometry B Clin Cytom, 2004. **60**(1): p. 1-13.
70. Shah, V.O., C.I. Civin, and M.R. Loken, *Flow cytometric analysis of human bone marrow. IV. Differential quantitative expression of T-200 common leukocyte antigen during normal hemopoiesis.* J Immunol, 1988. **140**(6): p. 1861-7.
71. Imhof, B.A. and M. Aurrand-Lions, *Adhesion mechanisms regulating the migration of monocytes.* Nat Rev Immunol, 2004. **4**(6): p. 432-44.
72. Ross, G.D. and V. Vetvicka, *CR3 (CD11b, CD18): a phagocyte and NK cell membrane receptor with multiple ligand specificities and functions.* Clin Exp Immunol, 1993. **92**(2): p. 181-4.
73. Hashimoto, D., J. Miller, and M. Merad, *Dendritic cell and macrophage heterogeneity in vivo.* Immunity, 2011. **35**(3): p. 323-35.
74. Sasmono, R.T., et al., *Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many other*

macrophage-specific transcripts and can transdifferentiate into macrophages in vitro in response to CSF-1. J Leukoc Biol, 2007. **82**(1): p. 111-23.

75. Sasmono, R.T., et al., *A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse.* Blood, 2003. **101**(3): p. 1155-63.
76. Sauter, K.A., et al., *The MacBlue binary transgene (csf1r-gal4VP16/UAS-ECFP) provides a novel marker for visualisation of subsets of monocytes, macrophages and dendritic cells and responsiveness to CSF1 administration.* PLoS One, 2014. **9**(8): p. e105429.
77. MacDonald, K.P., et al., *An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation.* Blood, 2010. **116**(19): p. 3955-63.
78. Austyn, J.M. and S. Gordon, *F4/80, a monoclonal antibody directed specifically against the mouse macrophage.* Eur J Immunol, 1981. **11**(10): p. 805-15.
79. Guilliams, M., et al., *Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF.* J Exp Med, 2013. **210**(10): p. 1977-92.
80. Schulz, C., et al., *A lineage of myeloid cells independent of Myb and hematopoietic stem cells.* Science, 2012. **336**(6077): p. 86-90.
81. Tepper, R.I., R.L. Coffman, and P. Leder, *An eosinophil-dependent mechanism for the antitumor effect of interleukin-4.* Science, 1992. **257**(5069): p. 548-51.
82. Fleming, T., M. Fleming, and T. Malek, *Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family.* J. Immunol., 1993. **151**(5): p. 2399-2408.
83. Chan, J., et al., *Macrophage lineage cells in inflammation: characterization by colony-stimulating factor-1 (CSF-1) receptor (c-Fms), ER-MP58, and ER-MP20 (Ly-6C) expression.* Blood, 1998. **92**(4): p. 1423-31.
84. Daley, J.M., et al., *Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice.* J Leukoc Biol, 2008. **83**(1): p. 64-70.
85. Shortman, K. and S.H. Naik, *Steady-state and inflammatory dendritic-cell development.* Nat Rev Immunol, 2007. **7**(1): p. 19-30.
86. Ribechini, E., P.J. Leenen, and M.B. Lutz, *Gr-1 antibody induces STAT signaling, macrophage marker expression and abrogation of myeloid-derived suppressor cell activity in BM cells.* Eur J Immunol, 2009. **39**(12): p. 3538-51.
87. Kurihara, T., et al., *Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor.* J Exp Med, 1997. **186**(10): p. 1757-62.
88. Geissmann, F., S. Jung, and D.R. Littman, *Blood monocytes consist of two principal subsets with distinct migratory properties.* Immunity, 2003. **19**(1): p. 71-82.
89. Serbina, N.V. and E.G. Pamer, *Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2.* Nat. Immunol., 2006. **7**(3): p. 311-7.
90. Lopes, M.F., A.C. Costa-da-Silva, and G.A. DosReis, *Innate immunity to Leishmania infection: within phagocytes.* Mediators Inflamm, 2014. **2014**: p. 754965.
91. Yurdakul, P., et al., *Compartment-specific remodeling of splenic micro-architecture during experimental visceral leishmaniasis.* Am J Pathol, 2011. **179**(1): p. 23-9.

92. Cervia, J.S., H. Rosen, and H.W. Murray, *Effector role of blood monocytes in experimental visceral leishmaniasis.* Infect Immun, 1993. **61**(4): p. 1330-3.
93. Mukhopadhyay, D., et al., *M2 Polarization of Monocytes-Macrophages Is a Hallmark of Indian Post Kala-Azar Dermal Leishmaniasis.* PLoS Negl Trop Dis, 2015. **9**(10): p. e0004145.
94. Leon, B., M. Lopez-Bravo, and C. Ardavin, *Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania.* Immunity, 2007. **26**(4): p. 519-31.
95. Fromm, P.D., et al., *Loss of TNF signaling facilitates the development of a novel Ly-6C(low) macrophage population permissive for Leishmania major infection.* J Immunol, 2012. **188**(12): p. 6258-66.
96. Steinbrink, K., et al., *Ineffective elimination of Leishmania major by inflammatory (MRP14-positive) subtype of monocytic cells.* Immunobiology, 2000. **202**(5): p. 442-59.
97. Sunderkotter, C., et al., *Resistance of mice to experimental leishmaniasis is associated with more rapid appearance of mature macrophages in vitro and in vivo.* J Immunol, 1993. **151**(9): p. 4891-901.
98. Beil, W.J., et al., *Differences in the onset of the inflammatory response to cutaneous leishmaniasis in resistant and susceptible mice.* J Leukoc Biol, 1992. **52**(2): p. 135-42.
99. Nagl, M., et al., *Phagocytosis and killing of bacteria by professional phagocytes and dendritic cells.* Clin Diagn Lab Immunol, 2002. **9**(6): p. 1165-8.
100. Rescigno, M., *Dendritic cells and the complexity of microbial infection.* Trends Microbiol, 2002. **10**(9): p. 425-61.
101. Tejle, K., et al., *Wild-type Leishmania donovani promastigotes block maturation, increase integrin expression and inhibit detachment of human monocyte-derived dendritic cells--the influence of phosphoglycans.* FEMS Microbiol Lett, 2008. **279**(1): p. 92-102.
102. Holm, A., et al., *Role of protein kinase C alpha for uptake of unopsonized prey and phagosomal maturation in macrophages.* Biochem Biophys Res Commun, 2003. **302**(4): p. 653-8.
103. Oghumu, S., et al., *Transgenic expression of CXCR3 on T cells enhances susceptibility to cutaneous Leishmania major infection by inhibiting monocyte maturation and promoting a Th2 response.* Infect Immun, 2015. **83**(1): p. 67-76.
104. Guilliams, M., et al., *IL-10 dampens TNF/inducible nitric oxide synthase-producing dendritic cell-mediated pathogenicity during parasitic infection.* J Immunol, 2009. **182**(2): p. 1107-18.
105. Aguilar Torrentera, F., et al., *Parasitic load and histopathology of cutaneous lesions, lymph node, spleen, and liver from BALB/c and C57BL/6 mice infected with Leishmania mexicana.* Am J Trop Med Hyg, 2002. **66**(3): p. 273-9.
106. Petritus, P.M., et al., *Leishmania mexicana induces limited recruitment and activation of monocytes and monocyte-derived dendritic cells early during infection.* PLoS Negl Trop Dis, 2012. **6**(10): p. e1858.
107. Childs, G.E., et al., *Inbred mice as model hosts for cutaneous leishmaniasis. I. Resistance and susceptibility to infection with Leishmania braziliensis, L. mexicana, and L. aethiopica.* Ann Trop Med Parasitol, 1984. **78**(1): p. 25-34.

108. DeKrey, G.K., H.C. Lima, and R.G. Titus, *Analysis of the immune responses of mice to infection with Leishmania braziliensis.* Infect Immun, 1998. **66**(2): p. 827-9.
109. Rocha, F.J., et al., *Cytokines, signaling pathways, and effector molecules required for the control of Leishmania (Viannia) braziliensis in mice.* Infect Immun, 2007. **75**(8): p. 3823-32.
110. Costa, D.L., et al., *CCR2 signaling contributes to the differentiation of protective inflammatory dendritic cells in Leishmania braziliensis infection.* J Leukoc Biol, 2016.
111. Italiani, P. and D. Boraschi, *From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation.* Front Immunol, 2014. **5**: p. 514.
112. Fortier, A.H., D.L. Hoover, and C.A. Nacy, *Intracellular replication of Leishmania tropica in mouse peritoneal macrophages: amastigote infection of resident cells and inflammatory exudate macrophages.* Infect Immun, 1982. **38**(3): p. 1304-8.
113. Scott, P., *Impaired macrophage leishmanicidal activity at cutaneous temperature.* Parasite Immunol, 1985. **7**(3): p. 277-88.
114. Hoover, D.L. and C.A. Nacy, *Macrophage activation to kill Leishmania tropica: defective intracellular killing of amastigotes by macrophages elicited with sterile inflammatory agents.* J Immunol, 1984. **132**(3): p. 1487-93.
115. David A. Leiby, N.K.-t., Phillip Scott, Carol A. Nacy, *Leishmaniasis*, in *Parasitic infections and the immune system*, F. Kierszenbaum, Editor. 1994, Academic Press, Inc.: San Diego. p. xii, 254 p.
116. Carswell, E.A., et al., *An endotoxin-induced serum factor that causes necrosis of tumors.* Proceedings of the National Academy of Sciences of the United States of America, 1975. **72**(9): p. 3666-70.
117. Aggarwal, B.B., et al., *Human tumor necrosis factor. Production, purification, and characterization.* J Biol Chem, 1985. **260**(4): p. 2345-54.
118. MacMicking, J., Q.W. Xie, and C. Nathan, *Nitric oxide and macrophage function.* Annu Rev Immunol, 1997. **15**: p. 323-50.
119. Vassalli, P., *The pathophysiology of tumor necrosis factors.* Annu Rev Immunol, 1992. **10**: p. 411-52.
120. Wallach, D., et al., *Tumor necrosis factor receptor and Fas signaling mechanisms.* Annu Rev Immunol, 1999. **17**: p. 331-67.
121. Probert, L., *TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects.* Neuroscience, 2015. **302**: p. 2-22.
122. Parameswaran, N. and S. Patial, *Tumor necrosis factor-alpha signaling in macrophages.* Crit Rev Eukaryot Gene Expr, 2010. **20**(2): p. 87-103.
123. M. Grell, M.C., *TNF and TNF superfamily*, in *Cytokines and Cytokine Receptors: Physiology and Pathological Disorders*, J.-P.R. Constantin A. Bona, Editor. 2001, CRC Press. p. 825.
124. Tetta, C., et al., *Tumour necrosis factor in serum and synovial fluid of patients with active and severe rheumatoid arthritis.* Ann Rheum Dis, 1990. **49**(9): p. 665-7.
125. Tektonidou, M.G. and F.N. Skopouli, *Visceral leishmaniasis in a patient with psoriatic arthritis treated with infliximab: reactivation of a latent infection?* Clin Rheumatol, 2008. **27**(4): p. 541-2.

126. Kritikos, K., et al., *An atypical presentation of visceral leishmaniasis infection in a patient with rheumatoid arthritis treated with infliximab.* J Clin Rheumatol, 2010. **16**(1): p. 38-9.
127. Fabre, S., et al., *Visceral leishmaniasis infection in a rheumatoid arthritis patient treated with infliximab.* Clin Exp Rheumatol, 2005. **23**(6): p. 891-2.
128. Balta-Cruz, S., et al., *Pseudolymphomatoid cutaneous leishmaniasis in a patient treated with adalimumab for rheumatoid arthritis.* Acta Derm Venereol, 2009. **89**(4): p. 432-3.
129. Garcia-Gonzalez, E., et al., *Mucocutaneous leishmaniasis in a patient treated with anti-TNF-alpha therapy.* Rheumatology (Oxford), 2012. **51**(8): p. 1517-8.
130. Franklin, G., J. Greenspan, and S. Chen, *Anti-tumor necrosis factor-alpha therapy provokes latent leishmaniasis in a patient with rheumatoid arthritis.* Ann Clin Lab Sci, 2009. **39**(2): p. 192-5.
131. Vieira, L.Q., et al., *Mice lacking the TNF receptor p55 fail to resolve lesions caused by infection with Leishmania major, but control parasite replication.* J Immunol, 1996. **157**(2): p. 827-35.
132. Wilhelm, P., et al., *Rapidly fatal leishmaniasis in resistant C57BL/6 mice lacking TNF.* Journal of immunology, 2001. **166**(6): p. 4012-9.
133. Fromm, P.D., et al., *Fatal Leishmaniasis in the Absence of TNF Despite a Strong Th1 Response.* Front Microbiol, 2015. **6**: p. 1520.
134. Schleicher, U., et al., *TNF-Mediated Restriction of Arginase 1 Expression in Myeloid Cells Triggers Type 2 NO Synthase Activity at the Site of Infection.* Cell Rep, 2016. **15**(5): p. 1062-75.
135. Klimpel, G.R., *Soluble factor(s) from LPS-activated macrophages induce cytotoxic T cell differentiation from alloantigen-primed spleen cells.* J Immunol, 1980. **125**(3): p. 1243-9.
136. Hirano, T., et al., *Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin.* Nature, 1986. **324**(6092): p. 73-6.
137. Burger, R., *Impact of interleukin-6 in hematological malignancies.* Transfus Med Hemother, 2013. **40**(5): p. 336-43.
138. Mihara, M., et al., *IL-6/IL-6 receptor system and its role in physiological and pathological conditions.* Clin Sci (Lond), 2012. **122**(4): p. 143-59.
139. Heinrich, P.C., et al., *Principles of interleukin (IL)-6-type cytokine signalling and its regulation.* Biochem J, 2003. **374**(Pt 1): p. 1-20.
140. Rose-John, S., *IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6.* Int J Biol Sci, 2012. **8**(9): p. 1237-47.
141. Greenhill, C.J., et al., *IL-6 trans-signaling modulates TLR4-dependent inflammatory responses via STAT3.* J Immunol, 2011. **186**(2): p. 1199-208.
142. Albrecht, L.J., et al., *Lack of Proinflammatory Cytokine Interleukin-6 or Tumor Necrosis Factor Receptor-1 Results in a Failure of the Innate Immune Response after Bacterial Meningitis.* Mediators Inflamm, 2016. **2016**: p. 7678542.
143. Xing, Z., et al., *IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses.* J Clin Invest, 1998. **101**(2): p. 311-20.

144. Wunderlich, F.T., et al., *Interleukin-6 signaling in liver-parenchymal cells suppresses hepatic inflammation and improves systemic insulin action.* Cell Metab, 2010. **12**(3): p. 237-49.
145. Trautwein, C., et al., *Acute-phase response factor, increased binding, and target gene transcription during liver regeneration.* Gastroenterology, 1996. **110**(6): p. 1854-62.
146. Mauer, J., et al., *Signaling by IL-6 promotes alternative activation of macrophages to limit endotoxemia and obesity-associated resistance to insulin.* Nat Immunol, 2014. **15**(5): p. 423-30.
147. Casella, G., et al., *IL4 induces IL6-producing M2 macrophages associated to inhibition of neuroinflammation in vitro and in vivo.* J Neuroinflammation, 2016. **13**(1): p. 139.
148. Korner, H., et al., *Distinct roles for lymphotoxin-alpha and tumor necrosis factor in organogenesis and spatial organization of lymphoid tissue.* Eur J Immunol, 1997. **27**(10): p. 2600-9.
149. Solbach, W., et al., *Suppressive effect of cyclosporin A on the development of Leishmania tropica-induced lesions in genetically susceptible BALB/c mice.* J Immunol, 1986. **137**(2): p. 702-7.
150. Wenzel, U.A., et al., *Leishmania major parasite stage-dependent host cell invasion and immune evasion.* FASEB J, 2012. **26**(1): p. 29-39.
151. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
152. Masmoudi, A., et al., *Old World cutaneous leishmaniasis: diagnosis and treatment.* J Dermatol Case Rep, 2013. **7**(2): p. 31-41.
153. Gumy, A., J.A. Louis, and P. Launois, *The murine model of infection with Leishmania major and its importance for the deciphering of mechanisms underlying differences in Th cell differentiation in mice from different genetic backgrounds.* Int J Parasitol, 2004. **34**(4): p. 433-44.
154. Lohoff, M., et al., *The Th1/Th2 paradigm and experimental murine leishmaniasis.* Int Arch Allergy Immunol, 1998. **115**(3): p. 191-202.
155. Kedzierski, L. and K.J. Evans, *Immune responses during cutaneous and visceral leishmaniasis.* Parasitology, 2014: p. 1-19.
156. Zimmermann, H.W., C. Trautwein, and F. Tacke, *Functional role of monocytes and macrophages for the inflammatory response in acute liver injury.* Front Physiol, 2012. **3**: p. 56.
157. Theodos, C.M., et al., *Role of tumor necrosis factor in macrophage leishmanicidal activity in vitro and resistance to cutaneous leishmaniasis in vivo.* Infect Immun, 1991. **59**(8): p. 2839-42.
158. Liew, F.Y., et al., *Tumour necrosis factor (TNF alpha) in leishmaniasis. I. TNF alpha mediates host protection against cutaneous leishmaniasis.* Immunology, 1990. **69**(4): p. 570-3.
159. Fonseca, S.G., et al., *TNF-alpha mediates the induction of nitric oxide synthase in macrophages but not in neutrophils in experimental cutaneous leishmaniasis.* Eur J Immunol, 2003. **33**(8): p. 2297-306.
160. Ritter, U., et al., *TNF controls the infiltration of dendritic cells into the site of Leishmania major infection.* Med Microbiol Immunol, 2008. **197**(1): p. 29-37.

161. Reis, E.A., et al., *Cytokine response signatures in disease progression and development of severe clinical outcomes for leptospirosis.* PLoS Negl Trop Dis, 2013. **7**(9): p. e2457.
162. Del Vecchio, G.C., et al., *Clinical significance of serum cytokine levels and thrombopoietic markers in childhood idiopathic thrombocytopenic purpura.* Blood Transfus, 2012. **10**(2): p. 194-9.
163. Bleriot, C., et al., *Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection.* Immunity, 2015. **42**(1): p. 145-58.
164. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets.* Nat Rev Immunol, 2011. **11**(11): p. 723-37.
165. Diehl, S. and M. Rincon, *The two faces of IL-6 on Th1/Th2 differentiation.* Mol Immunol, 2002. **39**(9): p. 531-6.
166. Roach, D.R., et al., *TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection.* J Immunol, 2002. **168**(9): p. 4620-7.
167. Davies, L.C., et al., *Tissue-resident macrophages.* Nat Immunol, 2013. **14**(10): p. 986-95.
168. Kolios, G., V. Valatas, and E. Kouroumalis, *Role of Kupffer cells in the pathogenesis of liver disease.* World J Gastroenterol, 2006. **12**(46): p. 7413-20.
169. Kaye, P.M. and L. Beattie, *Lessons from other diseases: granulomatous inflammation in leishmaniasis.* Semin Immunopathol, 2016. **38**(2): p. 249-60.
170. Li, W., B.P. Katz, and S.M. Spinola, *Haemophilus ducreyi-induced interleukin-10 promotes a mixed M1 and M2 activation program in human macrophages.* Infect Immun, 2012. **80**(12): p. 4426-34.
171. Roszer, T., *Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms.* Mediators Inflamm, 2015. **2015**: p. 816460.
172. Kaye, P. and P. Scott, *Leishmaniasis: complexity at the host-pathogen interface.* Nat Rev Microbiol, 2011. **9**(8): p. 604-15.
173. Brandonisio, O., et al., *Nitric oxide production by Leishmania-infected macrophages and modulation by cytokines and prostaglandins.* Parassitologia, 2001. **43 Suppl 1**: p. 1-6.
174. De Trez, C., et al., *iNOS-producing inflammatory dendritic cells constitute the major infected cell type during the chronic Leishmania major infection phase of C57BL/6 resistant mice.* PLoS Pathog, 2009. **5**(6): p. e1000494.
175. Shortman, K. and Y.J. Liu, *Mouse and human dendritic cell subtypes.* Nat Rev Immunol, 2002. **2**(3): p. 151-61.
176. Liang, B., et al., *Anti-interleukin-6 monoclonal antibody inhibits autoimmune responses in a murine model of systemic lupus erythematosus.* Immunology, 2006. **119**(3): p. 296-305.
177. Luig, M., et al., *Inflammation-Induced IL-6 Functions as a Natural Brake on Macrophages and Limits GN.* Journal of the American Society of Nephrology, 2015. **26**(7): p. 1597-1607.
178. Kling, J., et al., *Redundancy of interleukin-6 in the differentiation of T cell and monocyte subsets during cutaneous leishmaniasis.* Exp Parasitol, 2011. **129**(3): p. 270-6.

179. Moskowitz, N.H., D.R. Brown, and S.L. Reiner, *Efficient immunity against Leishmania major in the absence of interleukin-6.* Infect Immun, 1997. **65**(6): p. 2448-50.
180. Titus, R.G., et al., *Interleukin-6 deficiency influences cytokine expression in susceptible BALB mice infected with Leishmania major but does not alter the outcome of disease.* Infect Immun, 2001. **69**(8): p. 5189-92.
181. Wu, W., L. Weigand, and S. Mendez, *The IL-6-deficient mouse exhibits impaired lymphocytic responses to a vaccine combining live Leishmania major and CpG oligodeoxynucleotides.* Can J Microbiol, 2009. **55**(6): p. 705-13.
182. Kratochvill, F., et al., *TNF Counterbalances the Emergence of M2 Tumor Macrophages.* Cell Rep, 2015. **12**(11): p. 1902-14.
183. Kroner, A., et al., *TNF and Increased Intracellular Iron Alter Macrophage Polarization to a Detrimental M1 Phenotype in the Injured Spinal Cord.* Neuron, 2014. **83**(5): p. 1098-116.
184. Iwamoto, S., et al., *TNF-alpha drives human CD14+ monocytes to differentiate into CD70+ dendritic cells evoking Th1 and Th17 responses.* J Immunol, 2007. **179**(3): p. 1449-57.
185. Hunter, C.A. and S.A. Jones, *IL-6 as a keystone cytokine in health and disease.* Nat Immunol, 2015. **16**(5): p. 448-57.
186. Scheller, J., et al., *The pro- and anti-inflammatory properties of the cytokine interleukin-6.* Biochim Biophys Acta, 2011. **1813**(5): p. 878-88.
187. Wang, Y., et al., *Elevated maternal soluble Gp130 and IL-6 levels and reduced Gp130 and SOCS-3 expressions in women complicated with preeclampsia.* Hypertension, 2011. **57**(2): p. 336-42.
188. Camporeale, A. and V. Poli, *IL-6, IL-17 and STAT3: a holy trinity in auto-immunity?* Front Biosci (Landmark Ed), 2012. **17**: p. 2306-26.
189. Tanaka, Y. and E. Martin Mola, *IL-6 targeting compared to TNF targeting in rheumatoid arthritis: studies of olokizumab, sarilumab and sirukumab.* Ann Rheum Dis, 2014. **73**(9): p. 1595-7.
190. Nishimoto, N. and T. Kishimoto, *Inhibition of IL-6 for the treatment of inflammatory diseases.* Curr Opin Pharmacol, 2004. **4**(4): p. 386-91.
191. Fernando, M.R., et al., *The pro-inflammatory cytokine, interleukin-6, enhances the polarization of alternatively activated macrophages.* PLoS One, 2014. **9**(4): p. e94188.
192. Chomarat, P., et al., *IL-6 switches the differentiation of monocytes from dendritic cells to macrophages.* Nat Immunol, 2000. **1**(6): p. 510-4.
193. Costa, D.L., et al., *Serum cytokines associated with severity and complications of kala-azar.* Pathog Glob Health, 2013. **107**(2): p. 78-87.
194. Nylen, S., et al., *Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis.* J Exp Med, 2007. **204**(4): p. 805-17.
195. Ansari, N.A., S. Saluja, and P. Salotra, *Elevated levels of interferon-gamma, interleukin-10, and interleukin-6 during active disease in Indian kala azar.* Clin Immunol, 2006. **119**(3): p. 339-45.
196. Kumar, R. and S. Nylen, *Immunobiology of visceral leishmaniasis.* Front Immunol, 2012. **3**: p. 251.

197. Dos Santos, P.L., et al., *The Severity of Visceral Leishmaniasis Correlates with Elevated Levels of Serum IL-6, IL-27 and sCD14.* PLoS Negl Trop Dis, 2016. **10**(1): p. e0004375.
198. van der Poll, T., E.E. Zijlstra, and M. Mevissen, *Interleukin 6 during active visceral leishmaniasis and after treatment.* Clin Immunol Immunopathol, 1995. **77**(1): p. 111-4.
199. Murray, H.W., *Accelerated control of visceral Leishmania donovani infection in interleukin-6-deficient mice.* Infect Immun, 2008. **76**(9): p. 4088-91.
200. Mitani, H., et al., *Activity of interleukin 6 in the differentiation of monocytes to macrophages and dendritic cells.* Br J Haematol, 2000. **109**(2): p. 288-95.
201. Chomarat, P., et al., *TNF skews monocyte differentiation from macrophages to dendritic cells.* J Immunol, 2003. **171**(5): p. 2262-9.
202. Yimin and M. Kohanawa, *A regulatory effect of the balance between TNF-alpha and IL-6 in the granulomatous and inflammatory response to Rhodococcus aurantiacus infection in mice.* Journal of Immunology, 2006. **177**(1): p. 642-650.
203. Park, S.J., et al., *IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation.* J Immunol, 2004. **173**(6): p. 3844-54.
204. Coffer, P.J., L. Koenderman, and R.P. de Groot, *The role of STATs in myeloid differentiation and leukemia.* Oncogene, 2000. **19**(21): p. 2511-22.
205. Nelms, K., et al., *The IL-4 receptor: signaling mechanisms and biologic functions.* Annu Rev Immunol, 1999. **17**: p. 701-38.
206. Sanson, M., E. Distel, and E.A. Fisher, *HDL induces the expression of the M2 macrophage markers arginase 1 and Fizz-1 in a STAT6-dependent process.* PLoS One, 2013. **8**(8): p. e74676.
207. Hiasa, M., et al., *GM-CSF and IL-4 induce dendritic cell differentiation and disrupt osteoclastogenesis through M-CSF receptor shedding by up-regulation of TNF-alpha converting enzyme (TACE).* Blood, 2009. **114**(20): p. 4517-26.
208. Aderka, D., J.M. Le, and J. Vilcek, *IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice.* J Immunol, 1989. **143**(11): p. 3517-23.
209. Diehl, S., et al., *Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1.* Immunity, 2000. **13**(6): p. 805-15.
210. Yimin and M. Kohanawa, *A regulatory effect of the balance between TNF-alpha and IL-6 in the granulomatous and inflammatory response to Rhodococcus aurantiacus infection in mice.* J Immunol, 2006. **177**(1): p. 642-50.
211. Nakajima, K., et al., *A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells.* EMBO J, 1996. **15**(14): p. 3651-8.
212. Zhang, C., et al., *Interleukin-6/signal transducer and activator of transcription 3 (STAT3) pathway is essential for macrophage infiltration and myoblast proliferation during muscle regeneration.* J Biol Chem, 2013. **288**(3): p. 1489-99.
213. Herbert, D.R., et al., *Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology.* Immunity, 2004. **20**(5): p. 623-35.
214. Vats, D., et al., *Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation.* Cell Metab, 2006. **4**(1): p. 13-24.

215. Wermeling, F., et al., *Acute inflammation primes myeloid effector cells for anti-inflammatory STAT6 signaling.* Proc Natl Acad Sci U S A, 2013. **110**(33): p. 13487-91.
216. Bronte, V. and M.J. Pittet, *The spleen in local and systemic regulation of immunity.* Immunity, 2013. **39**(5): p. 806-18.
217. Phillips, R., et al., *Innate killing of Leishmania donovani by macrophages of the splenic marginal zone requires IRF-7.* PLoS Pathog, 2010. **6**(3): p. e1000813.
218. Wang, F., et al., *RIG-I mediates the co-induction of tumor necrosis factor and type I interferon elicited by myxoma virus in primary human macrophages.* PLoS Pathog, 2008. **4**(7): p. e1000099.
219. Polley, R., S. Zubairi, and P.M. Kaye, *The fate of heterologous CD4+ T cells during Leishmania donovani infection.* Eur J Immunol, 2005. **35**(2): p. 498-504.
220. Helft, J., et al., *Origin and functional heterogeneity of non-lymphoid tissue dendritic cells in mice.* Immunol Rev, 2010. **234**(1): p. 55-75.
221. Manh, T.P., et al., *Plasmacytoid, conventional, and monocyte-derived dendritic cells undergo a profound and convergent genetic reprogramming during their maturation.* Eur J Immunol, 2013. **43**(7): p. 1706-15.
222. Merad, M., et al., *The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting.* Annu Rev Immunol, 2013. **31**: p. 563-604.
223. Reizis, B., et al., *Plasmacytoid dendritic cells: recent progress and open questions.* Annu Rev Immunol, 2011. **29**: p. 163-83.
224. Sapoznikov, A., et al., *Organ-dependent in vivo priming of naive CD4+, but not CD8+, T cells by plasmacytoid dendritic cells.* J Exp Med, 2007. **204**(8): p. 1923-33.
225. Cella, M., et al., *Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization.* Nat Immunol, 2000. **1**(4): p. 305-10.
226. Boonstra, A., et al., *Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation.* J Exp Med, 2003. **197**(1): p. 101-9.
227. Salio, M., et al., *CpG-matured murine plasmacytoid dendritic cells are capable of in vivo priming of functional CD8 T cell responses to endogenous but not exogenous antigens.* J Exp Med, 2004. **199**(4): p. 567-79.
228. Mouries, J., et al., *Plasmacytoid dendritic cells efficiently cross-prime naive T cells in vivo after TLR activation.* Blood, 2008. **112**(9): p. 3713-22.
229. Di Pucchio, T., et al., *Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I.* Nat Immunol, 2008. **9**(5): p. 551-7.
230. Smelt, S.C., et al., *B cell-deficient mice are highly resistant to Leishmania donovani infection, but develop neutrophil-mediated tissue pathology.* J Immunol, 2000. **164**(7): p. 3681-8.
231. Joshi, T., et al., *B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against Leishmania donovani infections.* PLoS Pathog, 2009. **5**(5): p. e1000431.
232. Ouyang, W., et al., *Regulation and functions of the IL-10 family of cytokines in inflammation and disease.* Annu Rev Immunol, 2011. **29**: p. 71-109.

233. Murray, H.W., et al., *Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy.* Infect Immun, 2002. **70**(11): p. 6284-93.
234. Lang, T., et al., *Macrophage subsets harbouring Leishmania donovani in spleens of infected BALB/c mice: localization and characterization.* Cell Microbiol, 2000. **2**(5): p. 415-30.
235. Hey, Y.Y. and H.C. O'Neill, *Murine spleen contains a diversity of myeloid and dendritic cells distinct in antigen presenting function.* J Cell Mol Med, 2012. **16**(11): p. 2611-9.
236. Cavalcanti, A.S., et al., *Parasite load induces progressive spleen architecture breakage and impairs cytokine mRNA expression in Leishmania infantum-naturally infected dogs.* PLoS One, 2015. **10**(4): p. e0123009.
237. Cook, M.C., et al., *Generation of splenic follicular structure and B cell movement in tumor necrosis factor-deficient mice.* J Exp Med, 1998. **188**(8): p. 1503-10.
238. Kumar, R., et al., *Leishmania specific CD4 T cells release IFNgamma that limits parasite replication in patients with visceral leishmaniasis.* PLoS Negl Trop Dis, 2014. **8**(10): p. e3198.
239. Kar, S., C. Metz, and D. McMahon-Pratt, *CD4+ T cells play a dominant role in protection against New World leishmaniasis induced by vaccination with the P-4 amastigote antigen.* Infect Immun, 2005. **73**(6): p. 3823-7.
240. Subauste, C.S., A.H. Koniaris, and J.S. Remington, *Murine CD8+ cytotoxic T lymphocytes lyse Toxoplasma gondii-infected cells.* J Immunol, 1991. **147**(11): p. 3955-9.
241. Novais, F.O. and P. Scott, *CD8+ T cells in cutaneous leishmaniasis: the good, the bad, and the ugly.* Semin Immunopathol, 2015. **37**(3): p. 251-9.
242. Novais, F.O., et al., *Cytotoxic T cells mediate pathology and metastasis in cutaneous leishmaniasis.* PLoS Pathog, 2013. **9**(7): p. e1003504.
243. Rodrigues, O.R., et al., *Identification of regulatory T cells during experimental Leishmania infantum infection.* Immunobiology, 2009. **214**(2): p. 101-11.
244. Taher, J.H., et al., *Lymphocyte phenotyping, using cluster-of-differentiation (CD) markers, in young Iraqi children with visceral leishmaniasis.* Ann Trop Med Parasitol, 2009. **103**(8): p. 671-7.
245. Medina-Colorado, A.A., et al., *Splenic CD4+ T Cells in Progressive Visceral Leishmaniasis Show a Mixed Effector-Regulatory Phenotype and Impair Macrophage Effector Function through Inhibitory Receptor Expression.* PLoS One, 2017. **12**(1): p. e0169496.
246. Herrmann, M., et al., *Clearance of fetuin-A--containing calciprotein particles is mediated by scavenger receptor-A.* Circ Res, 2012. **111**(5): p. 575-84.
247. Martinez-Pomares, L., et al., *Fc chimeric protein containing the cysteine-rich domain of the murine mannose receptor binds to macrophages from splenic marginal zone and lymph node subcapsular sinus and to germinal centers.* J Exp Med, 1996. **184**(5): p. 1927-37.
248. Mildner, A. and S. Jung, *Development and function of dendritic cell subsets.* Immunity, 2014. **40**(5): p. 642-56.
249. Schleicher, U., et al., *NK cell activation in visceral leishmaniasis requires TLR9, myeloid DCs, and IL-12, but is independent of plasmacytoid DCs.* J Exp Med, 2007. **204**(4): p. 893-906.

250. Remer, K.A., et al., *Vaccination with plasmacytoid dendritic cells induces protection against infection with Leishmania major in mice.* Eur J Immunol, 2007. **37**(9): p. 2463-73.
251. Guillerey, C., et al., *Pivotal role of plasmacytoid dendritic cells in inflammation and NK-cell responses after TLR9 triggering in mice.* Blood, 2012. **120**(1): p. 90-9.
252. Kadowaki, N., et al., *Natural interferon alpha/beta-producing cells link innate and adaptive immunity.* J Exp Med, 2000. **192**(2): p. 219-26.
253. Palucka, A.K., et al., *Cross-regulation of TNF and IFN-alpha in autoimmune diseases.* Proc Natl Acad Sci U S A, 2005. **102**(9): p. 3372-7.
254. Angelot, F., et al., *Endothelial cell-derived microparticles induce plasmacytoid dendritic cell maturation: potential implications in inflammatory diseases.* Haematologica, 2009. **94**(11): p. 1502-12.
255. Taieb, J., et al., *A novel dendritic cell subset involved in tumor immunosurveillance.* Nat Med, 2006. **12**(2): p. 214-9.
256. Chan, C.W., et al., *Interferon-producing killer dendritic cells provide a link between innate and adaptive immunity.* Nat Med, 2006. **12**(2): p. 207-13.
257. Nakano, H., M. Yanagita, and M.D. Gunn, *CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells.* J Exp Med, 2001. **194**(8): p. 1171-8.
258. Pelayo, R., et al., *Derivation of 2 categories of plasmacytoid dendritic cells in murine bone marrow.* Blood, 2005. **105**(11): p. 4407-15.
259. Bauman, S.K., G.B. Huffnagle, and J.W. Murphy, *Effects of tumor necrosis factor alpha on dendritic cell accumulation in lymph nodes draining the immunization site and the impact on the anticryptococcal cell-mediated immune response.* Infect Immun, 2003. **71**(1): p. 68-74.
260. Catala, A., et al., *Anti-tumour necrosis factor-induced visceral and cutaneous leishmaniasis: case report and review of the literature.* Dermatology, 2015. **230**(3): p. 204-7.
261. Xynos, I.D., et al., *Leishmaniasis, autoimmune rheumatic disease, and anti-tumor necrosis factor therapy, Europe.* Emerg Infect Dis, 2009. **15**(6): p. 956-9.
262. Bogdan, C., et al., *Tumor necrosis factor-alpha in combination with interferon-gamma, but not with interleukin 4 activates murine macrophages for elimination of Leishmania major amastigotes.* Eur J Immunol, 1990. **20**(5): p. 1131-5.
263. Kong, F., et al., *Transcriptional Profiling in Experimental Visceral Leishmaniasis Reveals a Broad Splenic Inflammatory Environment that Conditions Macrophages toward a Disease-Promoting Phenotype.* PLoS Pathog, 2017. **13**(1): p. e1006165.
264. Holscher, C., et al., *Impairment of alternative macrophage activation delays cutaneous leishmaniasis in nonhealing BALB/c mice.* Journal of immunology, 2006. **176**(2): p. 1115-21.
265. Li, X., et al., *The absence of TNF permits myeloid Arginase 1 expression in experimental L. monocytogenes infection.* Immunobiology, 2017. **222**(8-9): p. 913-917.
266. Diefenbach, A., et al., *Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite.* Immunity, 1998. **8**(1): p. 77-87.

267. Niedbala, W., et al., *Effects of nitric oxide on the induction and differentiation of Th1 cells.* Eur J Immunol, 1999. **29**(8): p. 2498-505.
268. Stenger, S., et al., *Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase.* J Exp Med, 1996. **183**(4): p. 1501-14.
269. Murray, H.W. and C.F. Nathan, *Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral Leishmania donovani.* J Exp Med, 1999. **189**(4): p. 741-6.
270. Nashleanas, M., S. Kanaly, and P. Scott, *Control of Leishmania major infection in mice lacking TNF receptors.* J Immunol, 1998. **160**(11): p. 5506-13.
271. Ritter, U., et al., *The control of Leishmania (Leishmania) major by TNF in vivo is dependent on the parasite strain.* Microbes Infect, 2004. **6**(6): p. 559-65.
272. Umare, V., et al., *Effect of proinflammatory cytokines (IL-6, TNF-alpha, and IL-1beta) on clinical manifestations in Indian SLE patients.* Mediators Inflamm, 2014. **2014**: p. 385297.
273. Kishimoto, T., *Interleukin-6: from basic science to medicine--40 years in immunology.* Annu Rev Immunol, 2005. **23**: p. 1-21.
274. Dasgupta, B., et al., *Serial estimation of interleukin 6 as a measure of systemic disease in rheumatoid arthritis.* J Rheumatol, 1992. **19**(1): p. 22-5.
275. Dayer, J.M. and E. Choy, *Therapeutic targets in rheumatoid arthritis: the interleukin-6 receptor.* Rheumatology (Oxford), 2010. **49**(1): p. 15-24.
276. Koutsouraki, E., et al., *Increase in interleukin-6 levels is related to depressive phenomena in the acute (relapsing) phase of multiple sclerosis.* J Neuropsychiatry Clin Neurosci, 2011. **23**(4): p. 442-8.
277. Stelmasiak, Z., et al., *Interleukin-6 concentration in serum and cerebrospinal fluid in multiple sclerosis patients.* Med Sci Monit, 2000. **6**(6): p. 1104-8.
278. Narita, Y., et al., *The key role of IL-6-arginase cascade for inducing dendritic cell-dependent CD4(+) T cell dysfunction in tumor-bearing mice.* J Immunol, 2013. **190**(2): p. 812-20.
279. Kaur., J., et al., *IL-6 inhibition improves nitric oxide in rheumatoid arthritis.* Internet Journal of Rheumatology and Clinical Immunology, 2014.
280. Wolf, J., S. Rose-John, and C. Garbers, *Interleukin-6 and its receptors: a highly regulated and dynamic system.* Cytokine, 2014. **70**(1): p. 11-20.
281. O'Shea, J.J., M. Gadina, and R.D. Schreiber, *Cytokine signaling in 2002: new surprises in the Jak/Stat pathway.* Cell, 2002. **109 Suppl**: p. S121-31.
282. Osorio, E.Y., et al., *Progressive visceral leishmaniasis is driven by dominant parasite-induced STAT6 activation and STAT6-dependent host arginase 1 expression.* PLoS Pathog, 2012. **8**(1): p. e1002417.
283. Osorio, E.Y., et al., *Growth factor and Th2 cytokine signaling pathways converge at STAT6 to promote arginase expression in progressive experimental visceral leishmaniasis.* PLoS Pathog, 2014. **10**(6): p. e1004165.
284. Platanias, L.C., *Mechanisms of type-I- and type-II-interferon-mediated signalling.* Nat Rev Immunol, 2005. **5**(5): p. 375-86.
285. Qing, Y. and G.R. Stark, *Alternative activation of STAT1 and STAT3 in response to interferon-gamma.* J Biol Chem, 2004. **279**(40): p. 41679-85.

286. Kaye, P.M., et al., *The immunopathology of experimental visceral leishmaniasis.* Immunol Rev, 2004. **201**: p. 239-53.
287. Nikolic, T., et al., *Developmental stages of myeloid dendritic cells in mouse bone marrow.* Int Immunol, 2003. **15**(4): p. 515-24.

Appendix